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APPLICATION FOR LETTERS PATENT

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Title: ANTIBODY-MEDIATED DOWN-REGULATION
OF PLANT PROTEINS

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ANTIBODY-MEDIATED DOWN-REGULATION OF PLANT PROTEINS

RELATED APPLICATION

This application claims the priority of United States
5 Provisional Patent Application Ser. No. 60/093,587, filed
July 21, 1998.

FIELD OF THE INVENTION

This invention relates to the preparation and use of
nucleic acid fragments or genes encoding monoclonal
10 antibodies immunologically reactive to transit peptides
and the use of said genes to create transgenic plants
having altered protein levels.

BACKGROUND OF THE INVENTION

The modification of plants by genetic engineering
15 has been of considerable interest in recent years. To
date, several different plant species have been modified
by introducing new genes encoding proteins having
enzymatic activity. In essence, presence of these
enzymes encoded by transgenes results in the production
20 of proteins which can alter existing plant properties
and/or produce new and enhanced characteristics. When
said proteins are expressed, new or modified enzymatic
activities not previously endogenous to the plant can be
observed, or levels of proteins found therein can be
25 increased.

Another genetic approach that has been used to
modify plant species is down-regulation of a particular
plant protein. Down-regulation can result in either
partial or complete elimination of a particular enzymatic
30 activity of choice by decreasing overall steady state
levels of the protein associated therewith. A common
down-regulation approach used to modify a desired plant
enzyme level or activity is antisense RNA technology.
Antisense RNA results in down-regulation at the RNA
35 translational level. Down-regulation by antisense RNA,
as described in US Patent 5,107,065 to Calgene, has been
shown effective with a variety of plant genes as

described by Shimada et al., (1993) Theor. Appl. Genet. 86:665-672; Kull, et al., (1995) J. Genet. Breed. 49:67-76; Slabas and Elborough, (1997) WO 97/07222 to Zeneca, published 2/27/97; and Knutzon et al., (1992) Proc. Natl. Acad. Sci USA 89:2624-2628.

Another down regulation approach involves the use of ribozyme technology as described by Hasselhoff and Gerlach, (1988) Nature 342:76-79. Ribozyme technology, like antisense methodologies, also works at the RNA translational level and involves making catalytic RNA molecules which bind to and cleave the mRNA of interest. Ribozymes have recently been demonstrated as an effective method for the down-regulation of plant proteins as described in WO97/10328 to Ribozyme Pharmaceuticals and Dow AgroSciences, LLC, formally DowElanco.

Co-suppression, as described by Seymour et al., (1993, Plant Mol. Biol. 23:1-9) is another approach applicable for down-regulation of plant gene expression. At present, the precise mechanism of down-regulation via co-suppression is not known. However, it has been used extensively to produce transgenic plants having modified gene expression levels as described in Brusslan et al., (1993) Plant Cell 5:667-677; Vaucheret et al., (1995) Mol. Gen. Genet. 248:311-317; and Jorgensen et al., (1996) Plant Mol. Biol. 31:957-973.

As disclosed herein, Applicants have invented an alternative approach to down-regulate proteins in plants relying on the use of monoclonal antibodies (MAb) and functional fragments thereof, such as single chain antibodies (SCAb) that specifically recognize and bind transit peptides. As a result, steady-state levels of corresponding passenger proteins can be reduced. The proposed approach is further exemplified, as shown in the non-limiting examples, through down-regulation of maize stearoyl-ACP Δ -9 desaturase.

In many situations it will be desirable to modify an existing trait of a plant cell rather than introduce a new trait. Thus, one may wish to modify the overall

activity levels of a particular enzyme, provide for preferential accumulation of one allele as compared to another, one isozyme as compared to another or the like. In other instances, one may only wish to reduce the amount of expression of a protein encoded by a gene rather than inhibit expression entirely. It is therefore of interest to use the invention disclosed herein which will allow for directed modification of specific proteins in particular plant cells, plant tissues, or plants.

SUMMARY OF THE INVENTION

In the present invention, monoclonal antibodies have been generated against transit peptides that direct plant passenger proteins to organelles. Steady state levels of passenger proteins in plant cells can be altered by expressing antibodies which immunologically react to said transit peptides found attached to said passenger proteins in the precursor form.

One aspect of the invention is identification of transit peptides and genes and nucleic acids encoding the same. Information obtained from said genes and nucleic acids can be used to synthesize transit peptides.

Another aspect of the present invention relates to production of hybridoma cells lines producing monoclonal antibodies immunologically reactive to said transit peptides. Said antibodies can exhibit specificity for and high binding affinity to said transit peptides.

Another aspect of the disclosed invention is related to genes and nucleic acid fragments encoding monoclonal antibodies immunologically reactive to plant transit peptides. Said genes are obtained for the heavy chain and light chain portion of said antibodies.

Yet an additional aspect of the present invention relates to modifying genes encoding said monoclonal antibodies thus designing and expressing single chain antibodies (SCAb) derived therefrom. Said SCABs can exhibit binding specificity and antigen affinity similar

to said monoclonal antibodies but are encoded by a single gene.

An additional aspect of the present invention is the production of chimeric genes encoding monoclonal
5 antibodies and single chain antibodies (SCAb)
immunologically reactive to said transit peptides in combination with promoter regulatory elements and use of said chimeric genes within a plant cell. Expression of said genes can result in functional antibody proteins
10 which bind to transit peptides and thus lowers steady state levels of passenger proteins in a cell organelle.

Yet an additional aspect of the present invention is transformation of plant species disclosed herein with said chimeric genes.

15 Other aspects, embodiments, advantages, and features of the present invention will become apparent from the following specification.

Detailed Description of the Invention

20

The present invention relates to methods and compositions for obtaining transgenic plants having altered steady state passenger protein levels due to expression of antibodies being immunologically reactive
25 to transit peptides. The following phrases and terms are defined below:

By "altered steady state" is meant that the total level of a particular passenger plant protein having a transit peptide in a modified plant is different from
30 that of a normal or non-modified plant under similar conditions. Decreased steady state levels can be achieved by expressing antibodies immunologically reactive to said transit peptide.

35 By "antibody" is meant a protein molecule having functional activity comprising two identical polypeptide

chains of about 600 amino acid residues (usually referred to as heavy chains, H) covalently attached to each other by disulfide bonds, and two identical shorter polypeptide chains of about 220 amino acid residues (usually referred to as the light chains, L) each light chain being covalently attached to each other by disulfide bonds. The term antibody also includes immunologically-active fragments of the above described tetramer, as well as fragments, segments, proteolytically cleaved portions or recombinantly-prepared portions that still maintain antigen binding capacity as disclosed herein.

By "cDNA" is meant DNA that is complementary to and derived from a mRNA.

By "chimeric DNA construction" is meant a recombinant DNA containing genes or portions thereof from one or more species.

By "constitutive promoter" is meant promoter elements that direct continuous gene expression in all cell types and at all times (i.e., actin, ubiquitin, CaMV 35S, 35T, and the like).

By "cosuppression" is meant the introduction of a foreign gene having substantial homology to an endogenous gene, and in a plant cell can cause reduction in activity of the foreign gene and/or the endogenous protein product.

By "developmental specific" promoter is meant promoter elements responsible for gene expression at specific plant developmental stages, such as in early or late embryogenesis.

By "enhancer" is meant nucleotide sequence elements which can stimulate promoter activity such as those from maize streak virus (MSV) and alcohol dehydrogenase intron 1.

By "expression" as used herein, is meant the transcription and stable accumulation of the antibody RNA inside a plant cell.

By "foreign" or "heterologous gene" is meant a gene encoding a protein whose exact amino acid sequence is not normally found in the host cell, but is introduced therein.

5 By "functional" fragments, and "functional antibodies" is meant that the antibody or fragments thereof retain their ability to bind epitopes used in the immunization process and production of said antibody.

By "gene" is meant to include all genetic material
10 involved in protein expression including chimeric DNA constructions, genes, plant genes and portions thereof.

By "genome" is meant genetic material contained in each cell of an organism and/or virus.

By "indigenous" gene is meant the gene encoding the
15 precursor protein, passenger protein, or transit peptide as found in the native organism. Typically, the transit peptide and passenger protein are found together in the source from which the gene was isolated, although not required to be within the scope of the invention. A non-
20 limiting example of an indigenous gene would be the gene encoding the precursor protein maize Δ -9 desaturase, said gene being found in maize.

By "inducible promoter" is meant promoter elements which are responsible for expression of genes in response
25 to a specific signal, such as: physical stimuli (heat shock genes); light (RUBP carboxylase); hormone (Em); metabolites and stress.

By "mature protein" or "passenger protein" is meant the protein which is found after processing and passing
30 into an organelle. Passenger proteins are originally made in a precursor form that includes a transit peptide and said passenger protein. Upon entry into an organelle, the transit peptide portion is cleaved, thus leaving the "passenger" or "mature" protein. Passenger
35 protein are the proteins typically obtained upon purification from an homogenate, as described herein.

The sequence of passenger proteins can be determined as described herein. Passenger proteins can be indigenous in that they are naturally found in the organelle of interest. Passenger proteins can also be non-indigenous in that a plant may be transformed using a heterologous gene encoding a passenger protein not naturally found therein.

By "modified plant" is meant a plant wherein the protein levels or total protein specific activity levels of a passenger protein have been altered relative to that seen in a unmodified plant due to functional activity of an antibody.

By "nucleic acids" is meant to include all forms of DNA and RNA.

By "organelle" or "plant organelle" is meant to include chloroplasts, chromoplast, leucoplast, amyloplast, mitochondria, the nucleus, and the like.

By "phenotypic change" is meant to include alterations in steady state protein levels in a transformed or modified plant due to the expression of an antibody or fragments thereof binding to a organelle transit peptide.

By "plant" is meant a photosynthetic organism including both eukaryotes and prokaryotes.

By "precursor" protein is meant a protein having a transit peptide and a passenger protein covalently attached to each other. The passenger protein and transit peptide can be homologous to each other in that they are encoded in a manner isolated from nature. A non-limiting example could be the native maize transit peptide of Δ -9 desaturase covalently linked to the native maize Δ -9 desaturase passenger protein. In addition, the transit peptide and passenger protein can be heterologous to each other. A non-limiting example could be the native transit peptide for Δ -9 desaturase heterologously

attached to the passenger protein gene encoding chlorophyll a/b binding protein.

By "promoter regulatory element" is meant nucleotide sequence elements within a nucleic fragment or gene which control the expression of that nucleic acid fragment or gene. Promoter sequences provide the recognition for RNA polymerase and other transcriptional factors required for efficient transcription. Promoter regulatory elements are also meant to include constitutive, tissue-specific, developmental-specific, inducible promoters and the like. Promoter regulatory elements may also include certain enhancer sequence elements that improve transcriptional efficiency. In addition, promoters and promoter regulatory elements, as used herein, are meant to include subgenomic promoters found in +sense, single-stranded RNA viruses such as tobacco mosaic virus and the like.

By "tissue-specific" promoter is meant promoter elements responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (i.e., zein, oleosin, napin, ACP, globulin and like).

By "transgenic plant" is meant a plant expressing a chimeric gene introduced through transformation efforts.

By "transit peptide" or "signal peptide" is meant those amino acids that direct a passenger protein to a particular organelle as defined herein. It is within the scope of this invention that the transit peptide can be either indigenous or non-indigenous to the plant cell of interest. In addition, as further described herein, the transit peptide can be either homologous or heterologous to the passenger protein of interest in that it is natively found with said passenger protein as isolated from nature (homologous) or not (heterologous).

The present invention is directed to down regulation of organelle proteins by expression of antibodies or functional fragments thereof that are immunologically reactive to transit peptides. Down regulation can result

in decreased steady state levels of the passenger proteins or enzymatic activity levels of said passenger proteins. Passenger protein may be homologous or heterologous with respect to the transit peptide.

5 Furthermore, transit peptides may be homologous or heterologous with respect to passenger proteins. Both transit peptides and passenger proteins may be, either together or separately, indigenous or non-indigenous to the plant cell in which they are found.

10 In plants, chloroplast, mitochondria, the nucleus and other organelles as defined herein, use transit peptides to direct passenger proteins to the appropriate organelle. Transit peptides can be used as antigens, as disclosed herein, in the production of functional
15 antibodies immunoreactive thereto. Said functional antibodies can then be expressed within the cell whereby they can bind to said transit peptides and prevent accumulation of the passenger protein in the organelle and hence the cell. While chloroplast, mitochondria, and
20 the nucleus are the primary organelles found in plant tissue, cells are also likely to have other kinds of organelles including amyloplasts, chromoplast, leucoplast and the like, all of which use transit peptides for protein localization. Therefore, protein uptake can be
25 inhibited in plant organelles by practicing the invention as disclosed herein.

As further disclosed herein, expression of functional antibodies within plant cells which can bind transit peptides can result in decreased steady-state
30 levels of the passenger protein associated therewith when compared to non-transformed plants. Preferably, steady state levels of passenger proteins can be decreased from about 10% to about 90%; more preferably from about 50% to about 90%; with steady state levels of passenger proteins
35 being decreased about 90% compared to non-modified being most preferred.

As further described herein, amino acid, gene, and nucleic acid fragment sequence encoding transit peptides can be used to produced antibodies which can then be used in transgenic applications to produce plants having

5 altered steady-state protein levels. The invention is exemplified by determining the transit peptide sequence of maize stearoyl-ACP Δ -9 desaturase (Δ -9 desaturase) followed by producing, cloning, and expressing functional antibodies having an affinity to the same. In some

10 cases, transit peptide sequences can be determined by comparison of the gene and amino acid sequence encoding for the complete precursor protein as described herein. Preferably, genes and nucleic acid fragments encoding for passenger proteins and the use of said genes to determine

15 nucleic acid fragments and genes encoding transit peptides are isolated from plants. More preferred are those amino acid, gene, and nucleic acid fragments disclosed herein as SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7,

20 SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13 SEQ ID NO:14 and SEQ ID NO:15.

Hybridoma cell lines producing monoclonal antibodies can be obtained as disclosed herein. Said cell line most preferred is 10E10 (Mab-Tp1), which was deposited in

25 accordance with the terms of the Budapest Treaty at the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, on June 18, 1998 (ATTC Designation HB-12544). Monoclonal antibodies which are immunoreactive to the transit peptides

30 disclosed herein can be produced from hybridoma cell lines. Preferably, the amino acid sequence, gene sequence and nucleic acid fragments encoding precursor proteins, transit peptides, and passenger proteins can be determined. Most preferred are those amino acid, gene

35 and nucleic acid sequences disclosed herein as SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:25, SEQ ID

NO:26, SEQ ID NO:27 SEQ ID NO:28 SEQ ID NO:29, SEQ ID
NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:36, SEQ ID
NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID
NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID
5 NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID
NO:49, SEQ ID NO:50 and SEQ ID NO:51.

As disclosed herein, the invention relates to
identification and production of functional antibodies
against transit peptides which direct passenger proteins
10 to organelles. The amino acid sequence and hence the
nucleic acid sequence of a transit peptide can be
determined in a variety of ways available to the skilled
artisan. For example, passenger proteins of interest can
be purified using a variety of techniques as disclosed
15 herein. Once purified, amino terminal sequencing of said
protein can be determined. The gene encoding said
protein can then be cloned. Comparison of the amino acid
sequence determined by and from the cDNA to that obtained
from amino terminal sequence of the passenger protein
20 will allow determination of the transit peptide sequence.
In addition, many transit peptide sequences are available
in the art and can easily be obtained from GenBank
located at the National Center for Biotechnology
Information web site. In plants, transit peptides are
25 typically cleaved and degraded upon entry of the
passenger protein into the organelle of interest,
therefore, purification of transit peptides from plant
tissues is typically not possible.

Transit peptides of the present invention provide
30 intracellular transport to plastids and mitochondria.
The subject of transit peptides in plants has been
extensively reviewed by Keegstra et al., (1989) Cell,
56:247-253), which is incorporated herein by reference.
Typically, there is very little primary amino acid
35 sequence homology between different plant transit
peptides. In addition, even though passenger proteins

may have amino acid and nucleic acid sequence similarities between cultivars, lines, and species, transit peptides obtained therefrom may show very little amino acid or DNA sequence homology. Furthermore, the

5 length of transit peptides can vary, with some passenger proteins having transit peptides with as little as about 30 amino acid while other can be about 150 amino acids or longer. Nearly all transit peptide amino acid sequences nearly all start with Met-Ala at the amino terminus. The

10 carboxy terminus can often be determined by inspection in that cleavage most often occurs at or near the motif Val-Ala-Val-Val , Val-Ala-Ala-Val or variations thereof. In many cases, transit peptides may also contain further information for organelle targeting. Depending of the

15 source of the transit peptide, precursor proteins may display differences in import behavior, activity and efficiency. These differences are thought not to be only due solely to the transit peptide but also to passenger proteins and interaction associated therewith.

20 Additional descriptions of transit peptide characteristics in plants and mechanisms associated therewith can be found in Pfanner et al., (1988) Eur. J. Biochem, 175:205-212; Ko and Ko, (1992) J. Biol. Chem. 267, 13910-13916; Pfanner et al., (1988) TIBS 13:165-167;

25 Bascomb et al. (1992) Plant Microb. Biotechnol. Res. Ser. 1:142-163; and Bukau et al., (1996) Trends in Cell Biol. 6:480-486; which are incorporated herein by reference.

Transit peptides used to import proteins into the nucleus are also within the scope of this invention.

30 Antibodies can be made against said peptides and used to lower steady state levels of the passenger proteins as disclosed herein. Nuclear transit peptides are typically comprised of short polypeptide regions and can be classified as one of three distinct types. For example,

35 one type possesses a single short region enriched in basic amino acids. Another type is composed of two basic

regions separated by a spacer. Finally, a third type possesses both hydrophobic and basic amino acids.

Nuclear transit peptides in plants are further described in Silver (1991) Cell 64:489-497; Hicks et al., (1995)

- 5 Plant Physiol. 107:1055-1058; Ballas and Citovsky, (1997) Proc. Natl. Acad. Sci. USA, 94:10723-10728; Citovsky et al., (1994) Proc. Natl. Acad. Sci. USA (91): 3210-3214; Lyck et al., (1997) Planta, 202:117-125; Reiss et al., (1997) Mol. Gen. Genet. 253:695-702; Sakamoto et al.,
10 (1996) Plant J., 10:859-868; and Raikhel (1992) Plant Physiol. 100:1627-1632, which are incorporated herein by reference.

As further described herein, transit peptides once identified can be produced using a variety of methods.

- 15 Such methods can include heterologous expression in an appropriate system such as *E. coli*, Baculovirus, yeast and the like. The preferred method of peptide production is through chemical synthesis using either *t*-butyloxycarbonyl(*t*-BOC) or 9-fluorenylmethoxycarbonyl
20 (Fmoc) as described in Baraby and Merrifield (1980) In The Peptides Vol. 2 (E. Gross and Meienhofer, eds) pp.1-284, Academic Press, San Diego, CA; and Stewart and Young, (1984) Solid Phase Peptide Synthesis, 2nd ed. Pierce Chemical Co., Rockford, IL, which are incorporated
25 herein by reference.

- It is not necessary for the entire transit peptide to be used in antibody production to be within the scope of this invention. Generally, peptides with a length of about 10 to about 15 residues can be used; however,
30 peptides with as few as about 6 and up to about 150 amino acids or more are considered to be within the scope of the invention. Typically, polypeptide fragments rich in hydrophilic amino acids induce the best immunological response. Said hydrophilic amino acids include arginine,
35 aspartic acid, glutamic acid, lysine, serine, asparagine,

glutamine, glycine, and proline. However, polypeptide fragments with other amino acids can be used as well.

Antibodies bind to epitope sites based on amino acid/amino acid interactions and are thus highly specific
5 for individual epitope sites. However, as stated herein, there is a distinct lack of amino acid and DNA homology between different transit peptides found in plants. Therefore, the use of antibodies immunologically reactive thereto in one genus, species, cultivar, or line may or
10 may not be immunologically reactive to said transit peptides for the same passenger protein found in another genus, species, cultivar, or line. To ensure function, transit peptides from which the passenger protein of interest is derived are typically sequenced from a
15 variety of species, cultivars, or lines to see if common amino acid motifs can be determined. If so, these common amino acid motifs can be chosen for synthetic peptide and antibody production. If said sites do not exist, individual hybridoma lines for the transit peptide of
20 each passenger protein of interest may be required. This feature is especially important to consider in the production of hybrid crops, said crops being derived from transgenic and/or other parental lines.

Once a transit peptide is synthesized, it can be
25 purified prior to conjugation. A variety of purification methods are available to the artisan including gel filtration, dialysis, reverse phase high performance liquid chromatography, ion exchange chromatography and the like.

30 After the transit peptide is synthesized and purified it may be desirable to conjugate said peptide with an appropriate carrier protein to increase the immunogenic response. A carrier protein should be a good immunogen and have a sufficient number of amino acid
35 residues with reactive side chains for coupling to the synthetic peptide. Keyhole Limpet haemocyanin (KLH) is

commonly used because of its proven efficacy. Other proteins useful as carrier molecules include thyroglobulin, bovine serum albumin, tetanus toxin, and the like.

5 In addition to the choice of carrier protein, a method of coupling the synthetic peptide (antigen) to the carrier protein can also be selected. Most coupling methods rely on the presence of free amino, sulfhydryl, phenolic, or carboxylic groups. The chosen coupling
10 method can link the transit peptide to the carrier either through the carboxy- or amino terminal residue. A variety of coupling reagents are available to the artisan including glutaraldehyde, *Bis*-imido esters, *bis*-diaotized benzidine, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide and
15 the like.

 In accordance with the present invention, there is provided material suitable for eliciting an immunogenic response in a system of mammalian origin. Preferably, the mammal can be of rat, mouse, horse, goat, rabbit, and
20 the like. Most preferred is wherein a mouse is used for antibody production. Elicitation of said response is typically induced by immunization. Immunization protocols are well known in the art and can vary considerably yet remain effective as described in Current
25 Protocols in Immunology, Edited by John E. Coligan et al., John Wiley & Sons, Inc. (1991), which is incorporated herein by reference. Typically, about 1 μ g to 50 μ g of antigen are given in emulsions of about 1 mL to about 2 mL for a rat and about one-tenth as much for
30 mice. The first injection of antigen into a host animal may be subcutaneous, intramuscular, intraperitoneal or intravasular. Thereafter, about 1 to about 10 or more booster immunizations may be given at intervals of between about 2 and about 8 weeks. The route of
35 immunization may be varied according to protocol.

Immune response elicited following injection is typically monitored by performing western analysis to determine the level of antibody present in the animal's serum using techniques commonly available to one having
5 ordinary skill in the art of immunology. Injections of antigen are typically continued until the immunological response reaches a plateau of maximal response.

After immunization is complete, immune lymphocytes from the immunized animal are typically fused to myeloma
10 cells to generate hybridoma cell lines, which are immortal and produce monoclonal antibodies. Lymphocytes are generally taken from either lymph node tissue or spleen tissue and are fused to plasmacytoma cells, which are specialized myeloma cells available to the artisan
15 from the American Type Culture Collection, Manassas, VA. In a typical fusion, a suspension of lymphocyte cells is added to the myeloma cells in the presence of a fusogen such as polyethylene glycol (PEG). However, other methods of cell transformation are possible including
20 transformation of myeloma cells with viruses such as Epstein-Barr virus and the like.

After fusion, cells are harvested, diluted, and cultured for a week or more in separate wells containing the appropriate selective agent such as hypoxanthine,
25 aminopterin, and thymidine (HAT) which is capable of extinguishing non-fused cells. Cultured supernatants from said wells can be screened to determine hybridoma growth as well as determine the selectivity and affinity of a given antibody to the antigen. The cells may be
30 screened for the presence of antibodies capable of recognizing selected antigenic determinants by using methods available to the skilled artisan such as solid phase radioimmunoassay, enzyme-linked immunosorbent assays, western analysis, and the like. The cells so
35 selected can then be placed in individual wells as single cell colonies by limiting dilution. Feeder cells (e.g.

thymocytes, peritoneal exudate cells, and the like) may be added as necessary. After growing, supernatants therefrom can be characterized for antibody production by rescreening as described herein.

5 In accordance to the present invention, antibodies may be categorized by class or subclass, depending on which of a number of heavy chain constant domains they possess. Heavy chains are classified according to their constant region: IgA, IgD, IgE, IgG or IgM, with some
10 subclasses among them. Light chains are classified as either kappa or lambda. Categorization of antibodies may be performed using commercially available isotyping kits as disclosed herein.

Once hybridoma cell lines have been established and
15 antibodies have been produced and characterized, the antibodies can be purified for further analysis using a variety of techniques. Such techniques can include the use of Sepharose-protein G columns wherein the antibodies are bound followed by elution with 0.5 M ammonium acetate
20 (pH 3.0) and neutralization with 1 M Tris. Other purification techniques can include the use ammonium sulfate precipitation/size exclusion chromatography, affinity chromatography using protein-A Sepharose, as well as standard ion exchange chromatographic techniques
25 available to the artisan skill in the area of protein biochemistry. For purifying engineered antibodies, affinity tags can be encoded by the DNA so that amino acids are expressed which allow binding to a predetermined matrix. Such tags can include the 6X
30 Histidine motif which can bind to a Ni-Sepharose column, a c-myc tag, and the like.

The amino acid sequence of said antibody can be determined using a variety of methods available to the artisan. Such methods can involve direct protein
35 sequencing using a variety of techniques including Edman degradation, mass spectroscopy, nuclear magnetic

resonance, and the like. Other methods to determine amino acid sequence can involve the use of molecular biological techniques whereby the gene encoding said antibodies are cloned and sequenced, as disclosed herein.

- 5 The determination of amino acid sequence allows the antibody or derivatives thereof to be synthesized using a variety of peptide synthesis methods available to the artisan including solid phase t-butyloxycarbonyl (t-BOC), solid phase 9-fluorenylmethyloxycarbonyl (t-FMOC)
- 10 methods, as well as methods developed by Geysen et al., (1987, J. Immunol. Methods, 102:259-274); Houghton et al., (1985, Proc. Natl. Acad. Sci. USA, 82:5131-5135); and the like.

- In a preferred embodiment, antibodies can be
- 15 prepared in vitro from chromosomal DNA, cDNA or synthetic oligonucleotides using molecular biology techniques and heterologous expression systems such as *E. coli*, yeast, Baculoviruses, and the like. Cells or other source material containing DNA or RNA coding for the desired
- 20 antibody may be isolated. In some instances, it may be desirable to isolate the genomic DNA or mRNA (to generate cDNA) from such source material as described in Goldfein et al., (1987, J. Immunology, 138:940-944). When
- significant levels of nucleic acids are not available,
- 25 one can use polymerase chain reactions (PCR) to isolate nucleic acid fragments encoding the antibody of choice, as disclosed herein.

- If the amino acid sequence has been previously determined using methods disclosed herein or others
- 30 available to the skilled artisan, the genetic code can be used to reverse translate said amino acid sequence into a DNA sequence. A series of oligonucleotides ranging from about 20 to about 50 bases or more can then be synthesized in order to provide a series of overlapping
- 35 fragments. The synthesized oligonucleotides can be

annealed and ligated to create the gene encoding said antibody.

The complete genomic sequence of a particular antibody may be obtained by screening of a genomic or cosmid library with a probe. Probes can be considerably shorter than the entire gene sequence, but should be at least about 10 nucleotides, preferably at least about 15 nucleotides, more preferably at least about 20 or so nucleotides in length. Longer oligonucleotides up to the full length of the gene encoding the polypeptide of interest are also useful. Both DNA and RNA can be used as probes. In use, probes are typically labeled with ^{32}P , biotinylated, and the like in a manner that allows for detection thereof. Said probes are often incubated with single stranded DNA from the source of which the gene is desired. Hybridization, or the act of the probe binding to the DNA, is detected usually after binding using nitrocellulose paper or nylon membranes by means of the label on said probe. Hybridization techniques are well known to the person skilled in the art of molecular biology. Thus, antibody genes may be so isolated.

In a preferred embodiment, mRNA can easily be isolated from hybridoma cell lines expressing the antibody of interest using a variety of well known methods. Continually culturing said lines in conjunction with limiting dilutions as disclosed herein effectively results in a monoculture whereby cDNA obtained therefrom essentially encodes for only one antibody. After isotyping said antibody, use of oligonucleotide primers corresponding to constant regions found therein allows for the cloning thereof without necessarily proceeding through the steps common to other cloning procedures such as creation of libraries in phagemids, cosmids, and the like. Once cloned, the cDNA can be sequenced and the amino acid sequence can be determined via the genetic code.

One may engineer an antibody heavy chain or light chain variable region effectively homologous to the antibody heavy chain or light chain variable region of a known antibody. The term "effectively homologous" refers to the concept that the primary amino acid sequence of the variable region may be altered yet the antibody may retain a functional binding site with the capacity to bind to the same antigen or epitope. Accordingly, such variations and derivations are considered to be within the scope of the present invention. Antibodies so engineered may be experimentally determined using cross-blocking techniques wherein binding of one antibody prevents binding of a second antibody to the same epitope through either local or distant steric hindrance.

Modifications contemplated by the present invention include the addition, deletion, or conservative substitution of a limited number of various amino acids. Generally, an amino acid sequence is effectively homologous to a second amino acid sequence if at least about 70 percent, preferably at least about 80 percent, and most preferably, at least about 90 percent of the amino acid sequence of the variable portions of the two antibodies in question are homologous. Conservative substitutions are those exemplified such as glutamic acid and aspartic acid; valine, leucine, and isoleucine; asparagine and glutamine; threonine and serine; glycine and alanine; phenylalanine, tyrosine, and tryptophan; and lysine and arginine. Accordingly, such variations and derivations are considered to be within the scope of the present invention.

Binding affinities between the original and homologous antibody to the antigen of choice can also be used to determine "effective homology". Effectively homologous antibodies would be expected to have antibody:antigen binding constants that are at least within about 3 orders of magnitude compared to the

original antibody, and more preferably binding constants that are within about 2 orders of magnitude relative to the original antibody, with binding constants essentially the same as the original antibody being most preferred.

5 Determination of antibody binding constants is well known in the art.

Fragments of antibodies which maintain their ability to bind to the antigen are considered functional and thus considered to be within the scope of the present
10 invention. Said fragments include segments which are generated upon proteolytic cleavage or recombinantly prepared portions thereof. Thus, for example, portions of variable and constants domains of the antibody may be specifically altered or partially or completely omitted.
15 Nonlimiting examples of such recombinant fragments include Fab, F(ab)₂, and Fv fragments as described in U.S. Patent 4,642,334 to Moore and U.S. Patent 4,816,567 to Genentech. In addition, antibodies may be modified to form single chain Fv molecules as described in U.S.
20 Patent 4,946,778 to Genex Corp. and as disclosed herein. Said fragments and modifications thereto can also be created by manipulating genes encoding said fragments as disclosed herein. Recombinant constructs containing nucleic acid sequences encoding a functional antibody of
25 interest or fragments thereof and heterologous nucleic acid sequences may be prepared. By heterologous is meant any sequence which is not naturally found joined to the antibody sequence. Hence, by definition, a DNA sequence joined to a sequence not naturally found in a gene
30 encoding an antibody is considered to be heterologous.

Constructs may be designed to produce antibodies of interest or functional fragments thereof in either prokaryotic or eukaryotic cells. The expression of antibodies in a plant cells is of special interest.
35 Moreover, the nucleic acid sequences encoding said antibodies may be integrated into a plant host genome.

By transcribing and translating nucleic acid sequences encoding said antibodies or functional fragments thereof in a plant host, said plant is expected to exhibit properties wherein steady state passenger protein levels
5 associated with a gene transit peptide are lower than that observed in the non-transformed plant under similar conditions.

The scope of the invention as disclosed herein relates to decreased steady state levels of passenger
10 proteins being targeted to organelles. A wide variety of modifications may be made in numerous types of plants. Preferred monocotyledonous plants, for example, include rice, corn, wheat, barley, oats, rye and sorghum. Examples of preferred dicotyledonous plants
15 included canola, pea, soybean, sunflower, tobacco, cotton, sugar beet, petunia, tomato, broccoli, lettuce, apple, plum, orange, and lemon.

Modification to plants using the invention as disclosed herein may include varying the steady state
20 protein levels involved in fatty acid distribution of a fatty acid source, such as rapeseed, Cuphea, corn, soybean, canola or any oilseed crop of interest. Antibodies can also be made against the transit peptide of passenger proteins such as Δ -9 desaturase, palmitoyl-
25 ACP thioesterase, β -ketoacyl-ACP synthase, oleyl-ACP thioesterase, and the like. Antibodies can also be made against the transit peptide of proteins such as chlorophyll a/b binding proteins as a way to reduce chlorophyll content. Other organelle localized
30 proteins having transit peptides, of which antibodies can be made using said transit peptide as an antigen as disclosed herein, include: NADPH+ dependent glyceraldehyde-3-phosphate dehydrogenase, early light inducible protein, clip protease regulatory protease,
35 pyruvate orthophosphate dikinase, chlorophyll a/b binding protein, triose phosphate-3-phosphoglycerate

phosphate translocator, 5-enol pyruval shikimate-3-phosphate synthase, dihydrofolate reductase, thymidylate synthase, acetyl-coenzyme A carboxylase, Cu/Zn superoxide dismutase, cysteine synthase, rubisco
 5 activase, ferritin, granule bound starch synthase, pyrophosphate, glutamine synthase, aldolase, glutathione reductase, nitrite reductase, 2-oxoglutarate/malate translocator, ADP-glucose pyrophosphorylase, ferredoxin, carbonic anhydrase, polyphenol oxidase, ferredoxin NADP=
 10 oxidoreductase, platocyanin, glycerol-3-phosphate dehydrogenase, lipoxygenase, o-acetylserine (thiol)-lyase, acyl carrier protein, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, chloroplast-localized heat shock protein, starch phosphorylase,
 15 pyruvate orthophosphate dikinase, starch glycosyltransferase, and the like.

It is within the scope of the present invention that the steady state levels of passenger proteins can be decreased by transforming a plant cell with a gene
 20 encoding an antibody directed to the transit peptide associated with said passenger protein in a precursor protein form. It is also within the scope of this invention that transit peptides can be homologous or heterologous to the passenger protein as described
 25 herein. As further disclosed herein, transit peptides can be either indigenous or non-indigenous to the transformed plants. In a like manner, passenger proteins can be either indigenous or non-indigenous to the transformed plant cell.

30 Transit peptides and passenger proteins can be heterologous to each other in that they may not be associated in a manner found in nature. For example, the transit peptide from the small subunit of Rubisco (SSU) can be used to place a variety of proteins in the
 35 chloroplast of a plant cell thus causing proteins not normally found to accumulate therein. Antibodies

reactive against SSU transit peptide would be expected to prevent accumulation and decrease steady state levels of passenger proteins associated therewith relative to non-transformed controls.

5 To obtain high expression of heterologous genes in plants it may be preferred to reengineer said genes so that they are more efficiently expressed in the cytoplasm of plant cells. Maize is one such plant where it may be preferred to reengineer the heterologous gene(s) prior to
10 transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding antibodies for plant expression may involve reengineering of a heterologous gene for optimal expression.

15 One reason for the reengineering an antibody gene for expression in maize is to ensure the optimal G+C content of the native gene as well as eliminate any sequences mimicking or duplicating plant gene control sequences. The presence of some A+T-rich sequences
20 within the DNA of gene(s) introduced into plants (e.g., TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (e.g., polyadenylation
25 signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one aspect in the design of genes encoding an antibody gene for maize expression, more preferably referred to as plant
30 optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) is to generate a DNA sequence in which the sequence
35 modifications do not hinder translation.

The table below (Table 1) illustrates how high the G+C content is in maize. For the data in Table 1, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVector™ program (IBI, New Haven, CT). Intron sequences were ignored in the calculations.

Due to the plasticity afforded by the redundancy of the genetic code (i.e., some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of "minor" codons within a mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

In reengineering genes encoding an antibody for maize expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in Table 2. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The

primary codons preferred by the plant should be determined as well as the second and third choice of preferred codons. Afterwards, the amino acid sequence of the gene of interest is reverse translated so that the resulting nucleic acid sequence codes for exactly the same protein as the native gene wanting to be heterologously expressed. The new

Table 1. Compilation of G+C contents of protein coding regions of maize genes.

Protein Class ^a	Range %G+C	Mean %G+C ^b
Metabolic Enzymes (76)	44.4-75.3	59.0 (± 8.0)
Structural Proteins (18)	48.6-70.5	63.6 (± 6.7)
Regulatory Proteins (5)	57.2-68.9	62.0 (± 4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (± 7.2)
All Proteins (108)	44.4-75.3	60.8 (± 5.2)

^aNumber of genes in class given in parentheses.

^bStandard deviations given in parentheses.

^cCombined groups mean ignored in mean calculation.

DNA sequences are designed using codon bias information so that they correspond to the most preferred codons of the desired plant. The new sequences are then analyzed for restriction enzyme sites that might have been created by the modifications. The identified sites are further modified by replacing the codons with second or third choice with preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron 5' or 3' junctions, poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence.

Therefore, these blocks are also modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

It is preferred that the plant optimized gene(s) encoding an antibody contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The preferred codon usage for engineering genes for maize expression are shown in Table 2. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in pending PCT application WO 97/13402, which is incorporated herein by reference.

In order to design plant optimized genes, the amino acid sequence of the antibody of interest is reverse translated into a DNA sequence utilizing a non-redundant genetic code established from a codon bias table compiled for the gene sequences for the particular plant, as shown in Table 2. The resulting DNA sequence, which is completely homogeneous in codon usage, is further modified to establish a DNA sequence that besides having a higher degree of codon diversity, also contains strategically placed restriction enzyme recognition sites, desirable base composition, and a lack of sequences that might interfere with transcription of the gene or translation of the product mRNA.

In designing an antibody gene for optimal plant expression, it may be necessary to add nucleic acid sequences encoding amino acids that have been proven to increase the expression levels thereof. For example,

Schouten et al., (1996, Plant Molecular Biology, 30:781-793) have shown that the carboxy-terminal amino acid sequence KDEL increased antibody accumulation in transgenic tobacco. Other strategies for increasing expression of antibody genes have been explored by Owen et al., (1992, Chem. Ind., 11:406-408); Ma (1995, ACS Symp. Ser., 604:56-59); Schouten et al., (1997 FEBS Lett. 415:235-241); Conrad and Fiedler, (1994, Plant Mol. Biol. 26:1023-1030); Fiedler et al., (1997, Immunotechnology 3:205-216); van Engelen et al., (1994, Plant Mol. Biol. 26:1701-1710); Hiatt et al., (1989, Nature 342: 76-78); Firek et al., (1993, Plant Mol. Biol. 23:861-870); Ma et al., (1994, Eur. J. Immunol. 24:131-138), which are incorporated herein by reference.

In another aspect of the invention, genes encoding the antibodies are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and selection of transformed plant lines expressing mRNA encoding for said antibodies are expressed either by constitutive or inducible promoters in the plant cell. Once expressed, mRNA is translated into proteins, thereby incorporating amino acids of interest. Genes encoding antibodies expressed in plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, an inducible promoter, and the like. Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include acceleration of genetic material coated onto microparticles directly into cells (U.S. Patents 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, see U.S. Patent 5,177,010 to University of Toledo, 5,104,310 to

Texas A&M, European Patent Application 0131624B1, European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot, U.S. Patents 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot, 5 European Patent Applications 116718, 290799, 320500 all to Max Planck, European Patent Applications 604662, 627752 and US Patent 5,591,616 to Japan Tobacco, European Patent Applications 0267159, and 0292435 and U.S. Patent 5,231,019 all to Ciba Geigy, now Novartis, 10 U.S. Patents 5,463,174 and 4,762,785 both to Calgene, and U.S. Patents 5,004,863 and 5,159,135 both to Agracetus. Other transformation technology includes whiskers technology, see U.S. Patents 5,302,523 and 5,464,765 both to Zeneca. Electroporation technology has also been used 15 to transform plants, see WO 87/06614 to Boyce Thompson Institute, 5,472,869 and 5,384,253 both to Dekalb, WO9209696 and WO9321335 both to Plant Genetic Systems. Furthermore, viral vectors can also be used in produce transgenic plants expressing the protein of interest. 20 For example, monocotyledonous plant can be transformed with a viral vector using the methods described in U.S. Patents 5,569,597 to Mycogen and Ciba-Giegy, now Novartis, as well as U.S. Patents 5,589,367 and 5,316,931, both to Biosource. All of these patents and 25 publications on transformation are incorporated herein by reference.

As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method which provides 30 for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform Agrobacterium mediated transformation. In many instances, it will be desirable 35 to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the

right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation.

- 5 Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via
- 10 electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid
- 15 containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the *vir* genes are present.

Table 2. Preferred amino acid codons for proteins expressed in maize.

Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC

Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tyrosine	TAC/TAT
Stop	TGA/TAG
* The first and second preferred codons for maize.	

In some cases where Agrobacterium is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta et al., (PNAS USA (1980) 77:7347-7351 and EPO 0 120 515, which are incorporated herein by reference. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed Agrobacterium and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

For transformation of plant cells using Agrobacterium, explants may be combined and incubated with the transformed Agrobacterium for sufficient time to allow transformation thereof. After transformation, the agrobacteria are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage may not always be necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of

transformation technique, the antibody gene is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

In addition to numerous technologies for transforming plants, the type of tissue which is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue and the like. Almost all plant tissues may be transformed during cellular dedifferentiation using appropriate techniques described herein.

Another variable is the choice of a selectable marker. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which encode for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialophos); imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

In addition to a selectable marker, it may be desirable to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes which are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are

provided in K. Wising et al. (1988) Ann. Rev. Genetics, 22:421, which is incorporated herein by reference.

Preferred reporter genes include the beta-glucuronidase (GUS) of the uidA locus of *E. coli*, the chloramphenicol
5 acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after
10 said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the uidA locus of *E. coli* as described by Jefferson et al., (1987 Biochem. Soc. Trans. 15, 17-19) to identify transformed cells.

15 In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase
20 promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, see WO 97/13402 published April 17, 1997) and the like may be used. Plant promoter
25 regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters and tissue specific promoters.

30 Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences, and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary
35 for DNA function, although they can provide better expression or functioning of the DNA by affecting

transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-
5 intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan.

Constitutive promoter regulatory elements may also
10 be used thereby directing continuous gene expression in all cells types and at all times (e.g., actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the
15 leaves or seeds (e.g., zein, oleosin, napin, ACP, globulin, and the like) and these may also be used.

Promoter regulatory elements may also be active during a certain stage of the plants' development as well as active in plant tissues and organs. Examples of such
20 include but are not limited to pollen-specific, embryo specific, corn silk specific, cotton fiber specific, root specific, seed endosperm, fruit-specific, specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible
25 promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes); light (RUBP carboxylase); hormone (Em); metabolites; chemical; and stress. Other desirable transcription and
30 translation elements that function in plants may be used.

The present invention also contemplates the use of transformed plants which are selfed to produce an inbred plant. The inbred plant can produce seed containing genes encoding antibodies. The inbred lines can also be
35 crossed with other inbred lines to produce hybrids. Parts obtained from the regenerated plant, as flowers,

seeds, leaves branches, fruit and the like are covered by the invention provided that said parts contain genes encoding and/or express the antibody of interest. Progeny and variants, and mutants of the regenerated plants are also included within the scope of the invention.

In diploid plants, typically one parent may be transformed and the other parent maybe wild type. After crossing the parents, the first generation hybrids (F_1) can be selfed to produce second generation hybrids (F_2). Those plants expressing the highest levels of the antibody or having the desired phenotype can be chosen.

Another way to generate plants expressing active antibodies is to transform two plant lines, one with a gene encoding either a heavy chain or light chain and the other plant with the complimentary chain. The two or more plant lines can then be crossed to create progeny contain both heavy chain and light chain components. Functional antibodies can be obtained in this manner as described by Ma et al., (1995) Science 268:716-719.

Plant RNA viral based systems can also be used to express the antibody genes for the purposes as disclosed herein. In so doing, the gene can be inserted into the coat protein region of a suitable plant virus which will infect the host plant of interest. The antibody can then be expressed thus modifying steady state passenger protein levels. Plant RNA viral based systems are described in U.S. Patents 5,500,360 to Mycgoen Plant Sciences, Inc. and U.S. Patents 5,316,931 and 5,589,367 to Biosource Genetics Corp. which are incorporated herein by reference.

Standard and molecular biology techniques may be used to clone and sequence the toxins described herein. Additional information may be found in Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), Molecular

Cloning, A Laboratory Manual, Cold Spring Harbor Press, which is incorporated herein by reference.

The invention is illustrated in further details by the following non-limiting examples. The examples are
5 for the purpose of illustration only and are not to be construed as limiting the scope of the present invention.

EXAMPLE 1

10 ISOLATION AND CLONING OF STEAROYL-ACP Δ -9 DESATURASE (Δ -9 DESATURASE) FROM MAIZE

A cDNA clone encoding maize stearyl-ACP Δ -9 desaturase (Δ -9 desaturase) was obtained from a cDNA library derived from maize kernels of inbred CS608 (Mycogen Seeds, San
15 Diego, CA) that had been grown in a greenhouse and hand pollinated. The cDNA library was prepared from said kernels harvested at 20 days after pollination, hereinafter 20-DAP. Upon harvest, embryos were immediately collected, frozen on dry ice, and stored at -
20 70° C. RNA was extracted by grinding 2.5 g to a fine powder in liquid nitrogen. Afterwards, 10 mL of extraction buffer [50 mM Tris-HCl, pH 8.0, 4% para-amino salicyclic acid (Sigma Chemical Co), 1% tri-iso-propylnaphtalenesulfonic acid (Eastman Kodak Co.,
25 Rochester, NY), 10 mM DTT, and 10 mM sodium meta-bisulfite (Sigma Chemical Co., St. Louis, MO)] was added and the mixture was homogenized for 1 min using a TEKMAR TISSUMIZER (Tekmar Co., Cincinnati, OH). The homogenate was extracted with an equal volume of phenol equilibrated
30 with 0.1 M Tris-HCl, pH 8.0. Organic and aqueous phases were separated by centrifugation at 4° C. The aqueous phase was removed and extracted with an equal volume of chloroform/octanol (24:1). The supernatant was then transferred, centrifuged, transferred again, and a one-
35 half volume of 7.5 M ammonium acetate (pH 8.0) was added. RNA was then precipitated on ice for 30 min.

Precipitated RNA was collected by centrifugation and dissolved in 1 mL of diethylpyrocarbonate-treated water (0.1% v/v), hereinafter DEPC-water. One-half volume of 7.5 M ammonium acetate (pH 8.0) and two volumes of 100% ethanol were added and the RNA was allowed to precipitate at -20° C for 30 min. The precipitate was collected by centrifugation, washed in ice-cold 70% ethanol, air dried, and dissolved in 0.5 mL DEPC-treated water.

PolyA⁺ mRNA was purified on oligo dT-cellulose (Collaborative Biomedical Products, Bedford, MA) columns. Type 3 oligo-dT cellulose (0.1 g) was equilibrated in 5 mL of buffer 1 for 30 min, where buffer 1 was loading buffer with 0.5 M NaCl and loading buffer was 20 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 0.1% sodium lauryl sulfate (SDS). The column was washed with 3 volumes of DEPC-water, 3 volumes of wash buffer [0.1 N NaOH, 5 mM EDTA], 3 volumes of DEPC-water, and 5 volumes of buffer 1. The dissolved RNA pellet was heated at 65° C for 5 min, diluted 2x with buffer 2 [2x loading buffer] and then applied to the oligo-dT column. The flow through material was then collected, reheated, and reapplied to the column. Following, the column was washed with 10 volumes of buffer 1 followed by 10 volumes of buffer 3 [loading buffer having 0.1 M NaCl]. PolyA⁺ RNA was eluted with 3 volumes of elution buffer [10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% SDS] and collected in 0.5 mL fractions. RNA fractions were combined, buffered to 0.3 M sodium acetate pH 5.2, and precipitated at -20° C for 16 h after addition of 2.2 volumes of 100% ethanol. The precipitate was collected by centrifugation, washed with 70% ethanol, dried, and dissolved in 50 µL DEPC-treated water. This material was then repurified on a fresh oligo-dT column as described herein to produce highly-enriched polyA⁺ mRNA. RNA concentrations were determined by measuring OD_{260 nm}.

Five μg of polyA⁺ RNA was converted to cDNA and cloned into the LAMBDA UNI-ZAP vector using the Lambda ZAP-cDNA synthesis and cloning kit according to the manufacturers protocols (Stratagene, La Jolla, CA). The

5 resulting library had an original titer of 3.38×10^{10} plaque forming units/mL (pfu/mL), greater than 95% recombinants and an average insert size of 1.35 kb. The cDNA library was amplified according to Sambrook et al., (Molecular Cloning, A Laboratory Manual, 2nd Ed. (1989)

10 Cold Spring Harbor Laboratory Press) and had a titer of 6.0×10^6 pfu/mL. Total library cDNA was batch rescued and isolated as follows: 5 mL of XL1 Blue *E. coli* cells (Stratagene) at OD_{600nm} = 1.0 in 10 mM MgSO₄ were mixed with 8.3 μL (5×10^8 pfu) of amplified embryo cDNA library

15 phage-stock, and 100 μL EXASSIST helper phage (Stratagene) and incubated at 37° C for 20 min. Twenty-five mL of TY medium [8.0 g/L tryptone, 5.0 g/L yeast extract, and 2.5 g/L NaCl, pH 7.8] were added and cells were incubated at 37° C for 3 h while shaking.

20 Afterwards, bacterial cells were heat killed at 68° C for 15 min and the supernatant was recovered. Five hundred μL supernatant was mixed with 14.5 mL of SOLR cells (Stratagene) (OD_{600nm} = 1.5), incubated at 37° C for 15 min, added to 500 mL LB [10 g/L tryptone, 10 g/L NaCl,

25 and 5 g/L yeast extract containing 50 $\mu\text{g}/\text{mL}$ Ampicillin], and grown overnight. Afterwards, plasmid DNA was obtained by alkaline lysis/CsCl purification, according to Sambrook et al (Molecular Cloning, A Laboratory Manual, 2nd Ed. (1989) Cold Spring Harbor Laboratory

30 Press), which is incorporated by reference, and analyzed by agarose gel electrophoresis following digestion with EcoRI/XhoI. A smear ranging from 0.5 to 3.0 kb was observed following electrophoresis.

To isolate a clone encoding maize Δ -9 desaturase, a

35 DNA fragment was amplified using polymerase chain

reaction technology, hereinafter PCR, to produce a probe which could be used to isolate a full length cDNA. A 5' primer with 128-fold degeneracy and a 3' primer with 128-fold degeneracy, entered herein as SEQ ID NO:1 and SEQ ID NO:2, respectively, were synthesized on an Applied Biosystems High-Throughput DNA Synthesizer Model 394 (Foster City, CA). Double stranded cDNA was used as template. PCR amplification was performed as follows: 10 ng template DNA, 5 µL 10x reaction buffer, hereinafter 10X RB, [100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.01% (w/v) gelatin], 5 µL of 2 mM deoxyribose nucleotides triphosphate (dNTPs), 50 pmole primers (SEQ ID NO:1 and SEQ ID NO:2), 2.5 units AMPLITAQ DNA Polymerase (Perkin-Elmer, Norwalk, CT) and water for a total volume of 50 µL. A DNA Thermal Cycler (Perkin-Elmer Cetus Model #9600, Norwalk, CT) was programmed as follows: 96° C for 1 min; followed by [94° C (1 min), 37° C (2 min), and 72° C (3 min)] for 35 cycles; followed by 7 min at 72° C. A DNA product of 276 base pairs (bp) was obtained, sequenced as described, *infra*, and entered herein as SEQ ID NO:3.

The PCR fragment was cloned directly into the pBC vector (Stratagene, LaJolla, CA) and transformed into One Shot™ INVaF' competent cells (Invitrogen) according to manufacturers specifications. The DNA was extracted using the Qiawell Plasmid Purification System (Qiagen, Santa Clarita, CA) according to the manufacturer's instructions. Recombinant clones were sequenced by dideoxy chain termination using PRISM AMPLITAQ READY REACTION DYEDEOXY Terminator cycle sequencing kit #401384 according to the manufacturer (Perkin-Elmer Applied Biosystems Division, Foster City, CA). Samples were run on an ABI373A automated DNA sequencer (Perkin-Elmer, Applied Biosystems Division). DNA sequence analysis of SEQ ID NO:3 was performed using MACVECTOR v. 4.1.4 (Oxford Molecular, Campbell, KY), which gave theoretical

translations and alignments thus generating the amino acid sequence entered herein as SEQ ID NO:4.

The CS608 embryo cDNA library described herein was screened using a DNA fragment which was essentially SEQ ID NO:3. This fragment was used to obtain full length clones encoding maize Δ -9 desaturase. Probe DNA was obtained by digesting the cloned PCR fragment (SEQ ID NO:3) with the appropriate restriction enzymes. This material was then run on a preparative 1% agarose gel, the band was excised and the DNA was extracted with QIAEX (Qiagen). An $\alpha^{32}\text{P}$ -deoxyribocytosine triphosphate (dCTP)-labeled probe was generated using QUICKPRIME Random Labeling kit (Stratagene, LaJolla, CA) according to the manufacturer instructions using 5 μL of [$\alpha^{32}\text{P}$]-dCTP (3000 Ci/mmol, 10 $\mu\text{Ci}/\mu\text{L}$, DuPont, NEN Life Science Products, Boston, MA). Afterwards, the labeling reaction was applied to a NucTrap column (Stratagene) equilibrated with TE [10 mM Tris-HCl, pH 8.0, 1 mM EDTA]. Labeled DNA was eluted with 2 volumes of TE (400 μL each). The probe was heat denatured before being added to hybridization buffer as described herein.

Methods for phage titering, plating, coring and rescuing were as described in the LAMBDA ZAP II Library (Stratagene) instruction manual. The cDNA library was plated (100,000 pfu/plate) on five 22.5 x 22.5 cm NUNC assay plates (Nunc Inc. Roskilde, Denmark). Duplicate phage lifts were taken from each plate using 0.45 μm Hybond N+ nylon membranes (Amersham, Arlington Heights, IL). Following transfer of the phage to the filters, cells and phage were denatured and DNA was fixed to the membranes by autoclaving for 8 min at 125° C followed by UV-crosslinking using a STRATALINKER UV Crosslinker (Stratagene).

Prior to hybridization, filters were extensively washed in 2x SSC [1x SSC is 150 mM NaCl, 15 mM sodium

citrated, pH 7.0] 0.1% sodium dodecyl sulfate (SDS) at 65° C. Filter prehybridization was performed at 42° C for 1 h in 200 mL hybridization buffer containing 50% (v/v) formamide, 3x SSC, 10x Denhardt's solution [1x Denhardt's solution is 0.02% Ficoll (Type 400, Pharmacia), 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin], 0.1% (w/v) SDS, and 100 µg/mL sheared and denatured salmon sperm DNA. Afterwards, used hybridization buffer was replaced with 100 mL of fresh hybridization buffer containing labeled probe (final activity = 6.1×10^6 dpm/mL). Hybridization continued for 18-20 h at 42° C with gentle rotation. Afterwards, filters were washed twice at room temperature for 40 min in 100 mL of wash solution containing 0.1x SSC and 0.1% SDS, followed by 3 - 1 h washes with 1 L of the same at 60° C, followed by 2 - 1 h washes with 2 L of the same at 60° C. Filters were then exposed to Kodak XOMAT-AR Film (Eastman Kodak Company, Rochester, NY) with intensifying screens (Lightening Plus, DuPont CRONEX, DuPont, Wilmington DE) for 16 h at -70° C. Examination of films allowed the identification of positive plaques.

Some of the positive plaques were cored out and stored in 1 mL SM buffer [5.8 g/L NaCl, 2 g/L MgSO₄, 20 mM Tris-HCl, pH 7.5, 5 mL/L of 2% (w/v) gelatin] with 50 µL chloroform. Phage were plated for secondary screening using 50 µL of a 1:1000 dilution of the primary phage stock. Positive plaques from the secondary screening were cored out and stored in 500 µL of SM buffer. Phage from these stocks were then plated for tertiary screenings using amounts ranging from 5 µL of undiluted secondary stock to 20 µL of 1:100 dilution in SM buffer. All subsequent hybridizations were performed as described, *supra*. Isolates were rescued into phagemid form per the LAMBDA-ZAP II Library Instruction Manual (Stratagene). Rescued phagemid were plated by combining 200 µL SOLR cells (Stratagene) grown to OD_{600nm} = 0.5 to

1.0 with 50-100 μ L phagemid and incubating for 15 min at 37° C. Cells containing phagemid were streaked on LB agar containing Ampicillin (75 μ g/mL) and grown overnight at 37° C. DNA was extracted from 4 mL liquid cultures grown overnight at 37° C in TB [1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 0.17 M KH_2PO_4 , and 0.72 M K_2HPO_4] using the alkaline lysis/polyethylene glycol protocol described in the PRISM READY REACTION DYEDEOXY Terminator Cycle Sequencing Kit Protocol#401388 Rev. B (Perkin-Elmer, Applied Biosystems Division). A sequence of the full length maize Δ -9 desaturase cDNA and corresponding amino acid sequence is entered herein as SEQ ID NO:5 and SEQ ID NO:6, respectively.

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EXAMPLE 2

EXPRESSION OF THE GENES ENCODING MAIZE STEAROYL-ACP Δ -9 DESATURASE (Δ -9 DESATURASE) MATURE PROTEIN AND PRECURSOR PROTEIN

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The coding sequence of maize Δ 9 desaturase was cloned in expression vector pET9d DN using standard molecular cloning techniques. pET9d DN is a modified version of expression vector pET9d (Novagen Inc., Madison, WI) allowing subcloning of fragments in a unique NheI site downstream from the Shine & Dalgarno sequence and translation initiation ATG codon. To facilitate subcloning the cDNA clone (SEQ ID NO:5) was amplified with primers having the sequence entered herein as SEQ ID NO:7 and SEQ ID NO:8. Following amplification, the PCR product was digested with an excess of NheI, purified through agarose gel and directly ligated into vector pET9d DN which had been cut with NheI and dephosphorylated with calf intestinal phosphatase. The ligation was transformed into *E. coli* DH5 α cells and recombinant plasmids containing inserts were selected by miniprep screening and digestion with NheI. One clone

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with the appropriate sized insert in the correct orientation relative to the T7 promoter was identified by separate digestion with NheI, NcoI or XhoI. By subcloning fragment in the NheI site of pET9d DN, the putative precursor polypeptide was joined to the ATG codon downstream from the T7 promoter and bacterial Shine & Dalgarno sequence provided by pET9d DN. This NheI site changed the nucleotide sequence of the putative N-terminus of the precursor protein from SEQ ID NO: 57 to SEQ ID NO.: 58, which changed the encoded acid sequence SEQ ID NO.: 59 to SEQ ID NO.: 60. The insert of this clone was sequenced entirely and the clone was assigned number pDAB432D.

The precursor maize $\Delta 9$ desaturase was expressed in *E. coli* BL21(DE3) (Novagen Inc., Madison, WI). For small scale expression, 1 μ g of plasmid pDAB432D was transformed into 200 μ L CaCl₂ competent cells and plated on two LB plates containing kanamycin at 25 μ g/mL. Following overnight incubation at 37° C colonies were scraped off the plate and resuspended in 10 mL of LB Broth containing kanamycin (50 μ g/mL) and isopropyl- β -D-thiogalactoside (IPTG) at 1.0 mM. Cells were allowed to express proteins for 3 hr during vigorous shaking at 37° C. Cells were harvested by centrifugation at 3000 rpm for 10 min at 4° C. The cell pellet was frozen and thawed twice in dry ice-ethanol to improve cell lysis. Next, the cell pellet was resuspended in 1.0 mL of lysis buffer (10 mM Tris HCl pH 8.0, 1.0 mM EDTA, 150 mM NaCl, 0.1% Triton X 100, 100 μ g / mL DNaseI, 100 μ g / mL RNaseH, 1.0 mg / mL lysozyme) and incubated at 37° C until it was no longer viscous. Soluble proteins were separated from aggregated denatured proteins by centrifugation at 4° C for 10 min. The insoluble pellet was resuspended in about 300 μ L of the above lysis buffer. Both fractions had an approximate final volume of 0.5 mL.

Protein expression experiments were analyzed by electrophoresis of small aliquots (1.0 to 2.0 μ L) of the soluble and pellet fractions on 10-15% gradient SDS polyacrylamide Phast Gels (Pharmacia LKB Biotechnology, Piscataway, NJ). Protein bands were visualized by staining with Coomassie Brilliant Blue in the Phast Gel apparatus, and destained overnight by shaking in a small volume of 10% glycerol, 10% acetic acid. Molecular size of bands was determined by comparison to Bio-Rad Low Molecular Weight markers (Bio-Rad Laboratories, Hercules, CA). Western blot analysis was performed using antibodies produced as described herein specific to the mature portion of maize $\Delta 9$ -desaturase. Expression of plasmid pDAB432D in *E. coli* resulted in the production of a 42 kDa protein in the cell pellet, indicating that the precursor protein produced in *E. coli* was insoluble.

In production of mature $\Delta 9$ desaturase protein, the portion of the cDNA clone encoding the mature desaturase protein was subcloned into pETd Δ N. The amino terminus of the mature maize $\Delta 9$ desaturase protein determined as described herein. In order to express the mature protein, DNA corresponding to SEQ ID NO:11 was cloned into the expression vector pET9d Δ N which is a modified version of pET9d (Novagen, Inc.). The vector pET9d Δ N allowed subcloning of fragments in a unique NheI site downstream from the Shine and Dalgarno sequence and translation initiation ATG codon.

To facilitate subcloning, DNA corresponding to SEQ ID NO:5 was amplified with primers entered herein as SEQ ID NO:9 and SEQ ID NO:10. The DNA product had the sequence entered herein as SEQ ID NO:11 and encoded a protein having the amino acid sequence entered herein as SEQ ID NO:12. Following amplification, the PCR product was digested with an excess of NheI, gel purified through agarose gel and directly ligated into vector pET9d Δ N

which had been cut with *NheI* and dephosphorylated with calf intestinal phosphatase. The ligation was transformed into *E. coli* DH5 α cells, and recombinant plasmid containing inserts were selected by miniprep screening and digestion with *NheI*. Clones with the insert in the correct orientation relative to the T7 promoter were identified by separate digestions with *NcoI* or *XhoI*. The plasmid having the DNA construct in the correct orientation was named pDAB428. Expression of protein from said plasmid was performed in a manner similar to that described for plasmid pDAB432D.

EXAMPLE 3

PURIFICATION AND CHARACTERIZATION OF NATIVE AND *E. coli* EXPRESSED MAIZE STEAROYL-ACP Δ -9 DESATURASE (Δ -9 DESATURASE)

An *E. coli* produced supernatant containing mature maize Δ -9 desaturase, as described above, was adjusted to 25 mM sodium phosphate buffer (pH 6.0) and chilled on ice for 1 h. Afterwards, the resulting flocculant precipitant was removed by centrifugation. The ice incubation step was repeated twice after which the solution remained clear. The clarified solution was loaded onto a Mono S HR10/10 column (Pharmacia) that had been equilibrated in 25 mM sodium phosphate buffer (pH 6.0). Proteins bound to the column matrix were eluted using a 0-500 mM NaCl gradient over 1 h (2 mL/min; 2 mL fractions). Fractions containing Δ -9 desaturase activity as described herein were pooled and separated based on size using a Superose 12B HR16/50 (Pharmacia) column) at a flow rate of 1.2 mL/min that had been equilibrated in 50 mM Tris-HCl (pH 7.5). Fractions were collected (1.2 mL/fraction) and samples were assayed for the presence of Δ -9 desaturase protein via SDS-PAGE. Molecular size calculations were based on column calibration using the following standards: ribonuclease A (13.7 kDa);

ovalbumin (43.0 kDa); bovine serum albumin (67.0 kDa); aldolase (158.0 kDa); catalase (232.0 kDa); ferritin (440.0 kDa) and blue dextran (2,000 kDa).

The putative protein of interest was subjected to SDS-PAGE, blotted onto ProBlott membrane (Amersham), visualized with 0.02% amido black, excised, and sent to Harvard Microchem, (Boston, MA) for amino-terminal sequence analysis thus yielding the sequence entered herein as SEQ ID NO:13. Spectrophotometric analysis of the diiron-oxo component associated with the expressed protein (Fox et al., 1993, Proc. Natl. Acad. Sci. USA 90:2486-2490), as well as identification using a stain specific for nonheme iron (Leong et al., 1992, Anal. Biochem. 207:317-320) were also used to confirmed that the protein was mature Δ -9 desaturase.

The *E. coli* produced mature Δ -9 desaturase, as determined by amino terminal sequencing, was gel purified via SDS-PAGE and sent in the gel matrix to Berkeley Antibody Co. (Richmond, CA) for production of polyclonal antiserum in rabbits. Inoculations of antigen were initiated with 200 μ g protein followed by three boosting injections of 100 μ g each at three week intervals. Evaluations of antibody titers against mature Δ -9 desaturase were performed via western analysis using the ECL detection system (Amersham, Inc.; Arlington Heights, IL).

Polyclonal antiserum produced above were used for western analysis as follows: purified or partially purified proteins were subjected to SDS-PAGE analysis on a 10-20% polyacrylamide gel (Integrated Separation Systems, Natick, MA) using the method described by Laemmli (1970, Nature 277:680). Proteins were then transferred to nitrocellulose paper using a Pharmacia Semi-Dry Blotter and the nonspecific binding sites were blocked with BLOTTO (5% dry milk in phosphate buffered saline [PBS]/0.05% Tween-20). The membrane was incubated

in the polyclonal media (polyclonal antibody diluted 1:1000 in BLOTTO) for 1 h at room temperature while agitating on an orbital shaker. Afterwards, membranes were washed three times for 0.5 h each with excess BLOTTO followed by incubation for 0.5 h with a 1:2000 dilution of horseradish peroxidase linked goat-anti-rabbit polyclonal serum (BioRad, Hercules, CA). Following incubation, blots were washed three times for 10 min each with an excess of BLOTTO and then developed using the ECL Detection System (Amersham Inc.) as per the manufacturer's instructions.

Δ -9 desaturase was purified from corn kernels following homogenization using a Warring blender in 25 mM sodium phosphate buffer (pH 7.0) containing 25 mM sodium bisulfite and 2.5% polyvinylpyrrolidone. The crude homogenate was filtered through cheesecloth, centrifuged (10,000 x g) for 0.25 h and the resulting supernatant was filtered once more through cheesecloth. In some cases, the supernatant was fractionated via saturated ammonium sulfate precipitation at 20% (v/v). Upon centrifugation, the resulting supernatant was then precipitated with 80% (v/v) ammonium sulfate. Resulting pellets were dialyzed extensively against 25 mM sodium phosphate buffer (pH 6.0). After dialysis, the material was clarified via centrifugation and applied to a Mono S HR10/10 column (Pharmacia, Inc. Piscataway, NJ) equilibrated in 25 mM sodium phosphate buffer (pH 6.0). After extensive column washing, proteins bound to the column matrix were eluted using a 0-500 mM NaCl gradient over 1 h (2 mL/min; 2 mL fractions). Upon dialysis, this material was further fractionated by acyl carrier protein (ACP)-sepharose and phenyl Superose chromatography.

ACP-sepharose matrix was prepared as follows: ACP was purchased from Sigma Chemical Co. (St. Louis, MO) and purified by repeated precipitation at pH 4.1 followed by resolubilization (Rock and Cronan, 1981, Methods in

Enzymology 71:341-351). Attachment of ACP to cyanogen bromide-activated Sepharose 4-B beads was performed essentially as described by Pharmacia, Inc., in the package insert. After linkage and blocking of the remaining sites with glycine, the ACP-sepharose material was packed into a HR 5/5 column (Pharmacia, Inc.) and equilibrated in 25 mM sodium phosphate buffer (pH 7.0). Mono-S fractions identified as having Δ -9 desaturase activity were then loaded onto the ACP-Sepharose column as described previously (McKeon and Stumpf, 1982, J. Biol. Chem. 254:7116-7122; Thompson et al., 1991, J. Biol. Chem. 257:12141-12147). After extensive column washing, ACP-binding proteins were eluted using 1M NaCl.

Fractions containing Δ -9 desaturase activity obtained from the ACP-sepharose column were adjusted to 0.4 M ammonium sulfate (25 mM sodium phosphate, pH 7.0) and loaded onto a Pharmacia Phenyl Superose column (HR 10/10). Proteins were eluted by running a gradient (0.4 - 0.0 M ammonium sulfate) at 2 mL/min for 1 hr. Δ -9 desaturase activity typically eluted between 60- and 30 mM ammonium sulfate as determined by enzymatic and western analysis. Proteins were dialyzed extensively and concentrated using a Centricon-10 concentrator (Amicon, Inc., Beverly, MA). Samples were then blotted to PVDF membrane as described herein and sent to Harvard Microchem for amino terminal analysis. The amino terminal sequence is entered herein as SEQ ID NO:14.

Production of substrate for Δ -9 desaturase activity assays was performed as follows. Cells harboring a plasmid encoding acyl-ACP synthetase (Jackowski et al., 1994, J. Biol. Chem. 269:2921-2928) were grown, heat shock induced, and the acyl-ACP synthetase protein produced therein was partially purified essentially as described by Rock and Cronan, (1979, J. Biol. Chem. 254:7116-7122). [9,10 (n)- ^3H] stearic acid (specific

activity = 49 Ci/mmol) was custom synthesized by Amersham Life Sciences, Inc. Radioactive and non radioactive ACP-stearate was synthesized essentially as described by Rock et al., (1981, Methods in Enzymology 71:341-351).

5 Substrate production reactions were allowed to proceed for 16 h at 37° C before termination and purification. Non-radioactive substrate was made by using nonlabeled stearic acid. All reagents, unless otherwise specified, were obtained from Sigma Chemical Co.

10 Analysis of Δ -9 desaturase enzymatic activity was performed essentially as described by Thompson et al., (1991, Proc. Natl. Acad. Sci USA 88:2578-2582). Reactions were typically performed in triplicate and at
15 linearity. Non-radioactive ACP-stearate was spiked with ^3H -substrate and used at 60 μM final concentration (total volume = 500 μL ; total radioactivity = 5.1×10^4 dpm/sample). Reactions were allowed to proceed for 10 min at 25° C and were terminated by adding 0.5 mL of 12%
20 (w/v) trichloroacetic acid (TCA). After incubation for 5 min, the resulting precipitate was centrifuged and an aliquot of the remaining supernatant was removed (0.5 mL) and pH adjusted by adding 500 μL of 1M Tris (pH 8.0). The Δ -9 desaturase enzymatic activity was quantitated by
25 measuring the amount of ^3H released by liquid scintillation counting. A unit of activity was defined as the production of 1 μmole of oleate/min as determined by the release of $^3\text{H}_2\text{O}$ (McKeon and Stumpf, 1982, J. Biol. Chem. 257, 12141-12147). Testing of the maize purified
30 and *E. coli* expressed Δ -9 desaturase protein proved that both protein preparations had the appropriate desaturase enzymatic activity.

EXAMPLE 4

PRODUCTION OF SYNTHETIC Δ -9 DESATURASE TRANSIT PEPTIDE
(Δ -9TP) AND PRODUCTION OF POLYCLONAL ANTIBODIES SPECIFIC
THERE TO

5 As described herein, the maize Δ -9 desaturase gene
was cloned and the amino acid sequence of the putative
transit peptide (Δ -9TP) was determined and entered herein
as SEQ ID NO:15. This sequence information was sent to
Bio-Synthesis, Inc., (Lewisville, TX) for peptide
10 synthesis. Once received, the peptide was dissolved in
sterile water and aliquots thereof were analyzed by gel
electrophoresis on a 4-27% gradient gel (Integrated
Separation Science, Inc., Natick, MA). By comparing to
the molecular weight markers, the synthetic Δ -9TP was
15 determined to be about 4.0 kDa. The synthetic Δ -9TP was
then conjugated to Keyhole Limpet hemacyanin (KLH) and
injected into rabbits for polyclonal antibody production
by BioSynthesis, Inc. Serum was taken at 10 weeks and 13
weeks after the initial injection. Polyclonal antibodies
20 derived from this production were so named Pab-TP.

Immunospecificity of Pab-TP to the Δ -9TP peptide was
determined by Western blot analysis using *E. coli*
produced precursor (pDAB432D) and mature Δ -9 desaturase
protein (pDAB428), as well as the synthetically made Δ -
25 9TP polypeptide. Proteins were loaded onto a SDS-PAGE
gel as described herein and were then blotted to PVDF
membrane for western analysis as previously described.
In testing Pab-TP antibodies, it was observed that said
antibodies were immunologically reactive to both the
30 precursor Δ -9 desaturase and the Δ -9TP polypeptide but
did not recognize the mature protein. These data
indicated that Pab-TP antibodies were specific for the
transit peptide portion only of maize Δ -9 desaturase.

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EXAMPLE 5

PRODUCTION AND SCREENING OF HYBRIDOMA CELL LINES

Approximately 100 to 200 μ g of KLH conjugated Δ -9TP or Δ -9 desaturase mature protein was mixed with 1.0 mL of 1:1 mixture of Freund's Complete adjuvant and Freund's Incomplete adjuvant (Sigma Chemical Co.) for the initial immunizations. Samples were mixed only with Freund's incomplete adjuvant for subsequent immunization. Approximately 150 μ L of the antigen was injected intraperitoneally into individual Balb/c mice at two week intervals. After 5 injections, immunogenic response was determined by Western analysis of sera from said mice. Injections were continued if the immunogenic response was weak, otherwise, mice were used in the production of hybridoma cell lines.

The cell fusion protocol was based on the procedure described by Galfre and Milstein (1981, Methods in Enzymology 73:3-46). After the spleen was aseptically removed from an individual mouse, spleen cells were separated and washed twice with RPMI 1640 medium (CellgroTM, Fisher Scientific, Pittsburgh, PA). A viable cell count was determined after addition of an equal volume of Red Blood Cell Lysis Buffer (Sigma, St. Louis, MO), and 2 volumes of Trypan blue (Sigma, St. Louis, MO). Myeloma cells (X63Ag8) originally purchased from American Tissue Culture Collection (ATCC) were used as the fusion partner.

Myeloma cells were grown at a density of 7×10^5 cells/mL. Two days prior to cell fusion, cultures were diluted to 2×10^5 cells/mL and washed twice with RPMI 1640 medium. Approximately 10^8 spleen cells and 3.5×10^7 myeloma cells (a ratio of 3:1 spleen cells:myeloma cells) were used for each fusion. These cells were washed together once and the wash medium was discarded. One mL of pre-melted PEG 4000 (5.0 mg in 2.5 mL RPMI 1640 medium, 40° C) was added to the cell pellet. After 1 min,

20 mL of RPMI 1640 medium was gently added and the mixture was incubated at 37° C for 20 min. The medium was removed by centrifugation. Cell pellets were then resuspended in 200 mL of selection medium containing:

5 complete medium [500 mL RPMI 1640, 50 mL fetal bovine serum (Sigma Chemical Co.), 6 mL 200 mM L-glutamine (Cellgro™), 6 mL 100 mM sodium pyruvate (Cellgro™), 5000 units penicillin-streptomycin (Cellgro™)], 10% Origen Hybridoma Cloning Factor (HCF; Origen, Fisher

10 Scientific), and 1x HAT solution (hypoxanthine, aminopterin, and thymidine (Cellgro™). Cells were incubated for 1 h at 37° C and 200 µL thereof were distributed into each well of 96-well plates. After 10 to 14 days, media from each well were screened for

15 antibody-producing hybridomas.

ELISA techniques were used to screen for Δ-9TP specific monoclonal antibody (Mab) producing hybridoma cell lines. The assay involved first applying the antigen (10-50 ng of synthetic Δ-9TP or precursor protein

20 produced from pDAB432D) in 0.1 M sodium carbonate into wells of microtiter plates (Dynatech Laboratories, Chantilly, Virginia) and incubating overnight at 4° C. Afterwards, the excess antigen solution was discarded and the wells were blocked with 1% BSA (bovine serum albumin)

25 in PBS (phosphate buffer saline, Sigma) at 37° C for at least 1 h. The blocking solution was discarded, and 100 µL of hybridoma cell medium was added to each well. Plates were incubated at 37° C for at least 1 h, after which they were washed 3 times with wash solution (0.025%

30 Tween 20 in PBS). A 100 µL volume of a 1:1000 dilution of alkaline phosphatase conjugated anti-IgG + IgM antibodies (Kirkegaard & Perry, Gaithersburg, MD) in 1% BSA in PBS was added to each well. The plates were incubated at 37° C for 1 h and washed 3 times with the

wash solution. Finally, the phosphatase substrate p-NPP (para-nitrophenyl phosphate; Kirkegaard & Perry, Gaithersburg, MD) prepared according to the manufacturer's procedure was added to the wells. The color was allowed to develop for 10 to 30 min before the $A_{405\text{nm}}$ was determined using a plate reader (Molecular Devices, Sunnyvale, CA).

Cell lines determined positive by ELISA were increased by transferring the cells to 6-well plates containing fresh complete medium supplemented with 5% HCF. Once established, cell lines were re-screened by standard Western Blot analysis. Typically, 20 to 50 ng of precursor Δ -9 desaturase protein (pDAB432D) was applied to each well of a SDS polyacrylamide gel. After gel electrophoresis was completed, proteins were electroblotted onto Hybond-ECL nitrocellulose membrane (Amersham, Arlington Heights, IL) and western analysis was performed as described previously. Hybridoma cell medium obtained from test lines was used as the primary antibody. Secondary antibodies were typically goat-anti-mouse IgG conjugated to horseradish peroxidase (BioRad). Detection of immunological binding was performed using an ECL detection kit (Amersham) according to manufacturer's instruction.

Antibodies of interest were purified from the cell media using a Pure I antibody purification kit (Sigma). Two antibodies obtained from monoclonal cell lines (Mab-TP1 and Mab- Δ 9M) were further characterized by isotyping. Antibody isotype was classified using a Mouse Isotyping Kit (Boehringer Mannheim, Indianapolis, IN). Mab-TP1 and Mab- Δ 9M were determined to be IgG 2b Kappa Light Chain, and IgG1 Kappa Light Chain, respectively.

To determine binding specificity of the selected MABs, a Western blot analysis was performed using the

following as antigen: black mexican sweet corn (BMS) callus tissue, Type II corn callus tissue, maize seed extract, *E.coli* produced precursor protein (pDAB432D), and purified mature Δ -9 desaturase protein. Duplicate
5 blots were made and each blot was incubated with MAb-TP1, or MAb- Δ 9M. The result showed that MAb-TP1 could only recognize Δ -9 desaturase precursor protein and did not recognize mature Δ 9-desaturase or only other proteins in callus tissues or seeds. These data indicated that the
10 Mabs generated against Δ -9TP were specific thereto.

EXAMPLE 6

CLONING OF GENES FROM MONOCLONAL CELL LINES

Hybridoma cell lines of interest were grown in two 150 mL flasks containing 60 mL of hybridoma medium.
15 Seven days after inoculation, about 1×10^8 cells were harvested into 50 mL centrifuge tubes and pelleted by centrifugation at 800 rpm using a table-top centrifuge. The medium was discarded and the cells were washed twice with sterile PBS. Tubes containing washed cells were
20 placed on dry ice for 15 min prior to mRNA isolation.

PolyA+ mRNA was isolated using a Fast Track mRNA Isolation Kit (Invitrogen, Carlsbad, CA). Hybridoma cells were resuspended in 15 mL of lysis buffer provided therein. Cells were sheared by passing through a sterile
25 60 cc syringe fitted with an 18-21 gauge needle. This process was repeated 4 times. Lysates were incubated at 45° C for 15 min while shaking after which 950 μ L of 5M NaCl was added. Upon mixing thoroughly, the solution was passed through the syringe an additional 4 times in order
30 to cause DNA shearing. Seventy-five mg of powdered Oligo (dt) Cellulose was then added and the tube was rocked gently at room temperature for 1 h. Afterwards, lysates were centrifuge at 2000-4000 x g in a table top centrifuge for 5 min, supernatants were discarded, and

pellets were washed twice with binding buffer and low salt buffer as provided. Pellets were then resuspended in 800 μ L of low salt buffer. Cellulose suspensions were transferred into a spin column and washed 3 times by
5 centrifugation and by addition of low salt buffer. After the last centrifugation, pellets were resuspended in 200 μ L of Elution Buffer, the solution was collected by centrifugation and the process was repeated for a total volume of 400 μ L. To precipitate the RNA, 60 μ L of 2M
10 sodium acetate and 1000 μ L of absolute ethanol were added to the collected solution followed by freezing on dry ice. Following, tubes were centrifuged at 14,000 x g for 15 min and the mRNA pellet was resuspended in 20 to 40 μ L of sterile filtered water.

15 The mRNA obtained above was used to produce cDNA by reverse transcriptase reactions using cDNA Cycling[®] Kit (Invitrogen, Carlsbad, CA) according manufacturer's instructions. Typically, the total reaction volume was 20 μ L with 8 μ L of mRNA added thereto. Reactions were
20 started by adding mRNA and water followed by heating for 10 min at 65° C and then followed by cooling for 2 min at room temperature. Afterwards, RNase inhibitor, buffer, nucleotide triphosphates, sodium pyrophosphate and reverse transcriptase were added. Ingredients were mixed
25 and tubes were spun briefly before incubating at 42° C for 1 h. Tubes were then incubated at 95° C for 2 min before being placed on ice. cDNA synthesis was repeated again by adding additional reverse transcriptase followed by incubation for 1 h, followed by phenol extraction with 1
30 μ L 0.5 M EDTA, pH 8.0 and 20 μ L of Phenol/Chloroform/Isoamyl Alcohol (25:24:1). The tube was vortexed, centrifuged and the aqueous solution was recovered. To the aqueous solution was added 22 μ L of ammonium acetate and 88 μ L of absolute ethanol followed
35 by freezing on dry ice and collection of pellets by

centrifugation. Pellets were dissolved in sterile water and stored until further use.

Degenerate primers were designed and synthesized as disclosed herein. The 5' primers were designed to anneal to the signal peptide of the heavy (SEQ ID NO:16) or light chain genes (SEQ ID NO:17) based on published sequences (Kabat et al., 1987, Sequences of Proteins of Immunological Interest; 4th Ed. U.S. Department of Health and Human Services, Public Health Service, NIH). The 3' primers were designed to anneal to the constant region of the heavy (SEQ ID NO:18) and light chain genes (SEQ ID NO:19).

Genes of interest were obtained by PCR. The reaction (100 μ L total volume) was prepared using a PCR Core Kit (Boehringer Mannheim, Corp., Indianapolis, IN) and typically contained 0.5 μ g cDNA template and 100 pm of each primer. The heavy and light chain cDNAs were PCR amplified in separate experiments. PCR conditions were as follows: [95° C (1 min); 55° C (0.5 min); 72° C (0.5 min)] for 30 cycles followed by 1 cycle of 95° C (1 min); 55° C (0.5 min); 72° C for 2 min. PCR products were observed using agarose gel electrophoresis and ethidium bromide staining. Amplification of the heavy and light chains resulted in DNA fragments of about 730 bp each entered herein as SEQ ID NO:24 and SEQ ID NO:21, respectively. Products were then digested with the NheI, cloned into pBlueBac1 (Invitrogen, Carlsbad, CA) and transformed into DH5 α strain of *E. coli* using standard procedures. Clones were recovered and digested with NheI to verify the DNA insert. Plasmid DNA was produced, isolated, and sequenced as previously described using several external and internal primers. The DNA encoding the light chain (SEQ ID NO:50) was modified as follows: a 5' primer, entered herein as SEQ ID NO:20, was designed to replace the native mouse signal peptide with a

Baculovirus gp67 and (p67) leader (Murphy et al., 1993, Protein Expression and Purification, 4:349-357). SEQ ID NO:50 was used as the 3' primer. After performing PCR as described herein, the DNA was digested with *Xba* I and *Not* I enzymes and inserted into a Baculovirus transfer plasmid, pAcMP3 (PharMingen, San Diego, CA). The cloned plasmid (AcMP3/TpLCp67) was transformed into the DH5 α strain of *E. coli* (Gibco, Gaithersburg, MD). Several clones were recovered on the selection plates. Plasmid DNA was isolated from a selected clone and re-sequenced. Said sequence is entered herein as SEQ ID NO:49. The DNA encoding the hypervariable region of said clone is entered herein as SEQ ID NO:22. Comparison of sequence information indicated that the cloned Kappa chain was the authentic light chain of the Mab- Δ -9TP1 gene and not the endogenous non-functional light chain of myeloma cells (X63Ag8).

The DNA encoding the heavy chain (SEQ ID NO:24), obtained by using PCR primers SEQ ID NO: 16 and SEQ ID NO:18, was purified, digested with *Xba* I and *Not* I and ligated into pAcMP3. Sequencing data indicated that said clone was an authentic IgG2B heavy chain gene. The gene was re-amplified by PCR using primers with a sequence according to SEQ ID NO:23 and SEQ ID NO:18 to add a p67 Baculovirus leader, cloned into the AcMP3 Baculovirus transfer plasmid (AcMP3/TpHCp67) and transformed into *E. coli* as described herein. Colonies were screened and an appropriate clone was sequenced. The sequence (SEQ ID NO:51) matched that previously determined for the Mab-TP1 heavy chain. Comparison to known heavy chains revealed that the two CH1 sequences were identical indicating that the cloned heavy chain gene is an authentic IgG2B sequence. The sequence of the hypervariable region is entered herein as SEQ ID NO:25.

35

EXAMPLE 7

GENERATION OF RECOMBINANT BACULOVIRUS EXPRESSING Mab-TP1

SF9 (Invitrogen) cells were cultured at 27° C in spinner flasks with Sf900II (Gibco, Gaithersburg, MD) serum free complete medium supplemented with penicillin/streptomycin/fungizone at 1/2 rate. Cells were split to 3×10^5 cells/mL every four days. SF9 cells were seeded at 8×10^5 cells/well in a six well plate and allowed to attach for 1 h at 27° C. The medium was discarded and cells were washed twice with fresh medium without antibiotics. After the last wash, 1.5 mL of fresh medium without antibiotics was added. In a polystyrene tube, 0.1 µg of linearized parental Baculovirus DNA (Baculogold, PharMingen, San Diego, CA) was mixed with 0.5 µg of the transfer vector (AcMP3/TpLCp67 or AcMP3/TpHCp67 plasmid), followed by transfection mix as described in the standard cationic liposome mediated transfection protocol as provided by CLONTECH (Palo Alto, CA). Transfection medium was harvested after four days and samples of medium and cell pellet were analyzed for protein expression by western analysis as described herein. A 1:1000 dilution of goat anti-mouse FAb-specific IgG conjugated to horseradish peroxidase (Sigma Chemical Co.) was used with a ECL detection kit (Amersham). The results indicated that light chain and heavy chain proteins could be produced and recognized by appropriate antibodies, thus authenticity was confirmed.

To determine if functional antibodies could be produced, recombinant virus, AcMP3/TpLCp67 and AcMP3/TpHCp67 were co-infected in SF9 cells. The cell medium was harvested and used as the primary antibody in Western analysis using precursor Δ -9 desaturase (pDAB432D) as antigen. The expected sized band (42 kDa)

was observed indicating that the recombinant antibody produced was indeed functional.

EXAMPLE 8

5 CONSTRUCTION OF THE SINGLE CHAIN ANTIBODY-TP1 (SCAB-TP1) GENE

Generation of the *SCAb-Tp1* gene was accomplished via a two step PCR reaction using the PCR core kit (Boehringer Mannheim). Standard reactions contained
10 200µM dNTPs, 100 pM primer, SEQ ID NO:24 or SEQ ID NO:49 as template (0.5 µg), PCR buffer and 4 units of Taq polymerase as described previously. A sample of each reaction was analyzed in a 1% agarose gel. Approximately equal molar amounts of light and heavy chain product were
15 then added to a second reaction using the same conditions as above except that different primers were used. The resulting PCR products were then isolated and purified from 1% agarose gels using the Qiaex II kit (Qiagen).

Amplification of the heavy chain variable region of
20 SEQ ID NO:24 (which is SEQ ID NO:25) with primers according to SEQ ID NO:23 and SEQ ID NO:26 resulted in a PCR product of ~450 bp entered herein as SEQ ID NO:29. Amplification of the light chain variable region of SEQ ID NO: 21 (which is SEQ ID NO:22) with primers according
25 to SEQ ID NO:27 and SEQ ID NO:28 resulted in PCR product of ~440 bp entered herein as SEQ ID NO:30. A sewing reaction using equal molar amounts DNA according to SEQ ID NO:29 and SEQ ID NO:30 using primers according to SEQ ID NO:23 and SEQ ID NO:28 yielded the *SCAb-Tp1* gene
30 product of ~830 bp and having a DNA and amino acid sequence entered herein as SEQ ID NO:31 and SEQ ID NO:32, respectively. The DNA was then digested with Xba I and Not I and ligated into the AcMP3 Baculovirus transfer vector (PharMingen). A positive clone was identified and
35 sequenced (SEQ ID NO:31), a c-myc tag (SEQ ID NO:33) was

added to the 3' end of the gene and the p67 leader sequence (SEQ ID NO:34) was added at the 5' terminus.

The SCAb-TP1 gene (SEQ ID NO:31) was reconstructed by PCR using the reaction conditions described above.

5 Amplification of DNA according to SEQ ID NO:22 with primers according to SEQ ID NO:35 and SEQ ID NO:36 resulted in a product of ~450 bp, having the sequence entered herein as SEQ ID NO:40. Amplification of DNA according to SEQ ID NO:25 with primers according to SEQ
10 ID NO:37 and SEQ ID NO:38 (TABLE V) resulted in a product of ~440 bp having a DNA sequence according to SEQ ID NO:41. Approximately equal molar amounts of each product (SEQ ID NO:40 and SEQ ID NO:41) were added to another reaction with primers according to SEQ ID NO:35 and SEQ
15 ID NO:38 yielding a product of ~846 bp (SEQ ID NO:42). An additional PCR reaction was performed with DNA according to SEQ ID NO:42 using the primer pair having a DNA sequence according to SEQ ID NO:35 and SEQ ID NO:39 to add a stop codon to the 3' end of the gene. The
20 ~849bp product (SEQ ID NO:43) was isolated from a 1% agarose gel, digested with Xba I/Not I and ligated into the AcMP3 transfer vector (AcMP3/TPSCegt). An appropriate clone was identified and sequenced.

AcMP3/TPSCegt and BacPAK6 Bsu36 I linear parental
25 DNA (Clontech) were co-transfected into SF9 insect cells to create recombinant virus. Cationic liposome mediated transfection was performed as described in the instructions included with the BacPAK6 DNA. The transfection media was harvested after 48 h and 0.5 mL
30 was used to infect a 175cm² flask containing 2 x 10⁷ SF9 cells to amplify the recombinant virus. Western analysis performed as described herein identified a ~28kD protein in infected cells using an anti-HIS antibody (Qiagen, Inc., Chatsworth, CA). SCAb-Tp1 produced in the
35 Baculovirus system was compared to the native MAb-Δ-9TP1 in Western blots using E. coli expressed Δ-9 desaturase

precursor (pDAB432D) as antigen. These blots indicated no difference in the binding pattern between the native MAb TP1 and the recombinant SCAb-TP1.

The SCAb-TP1 gene was rebuilt to facilitate cloning into the pDAB439 plant transformation vector. PCR reactions were performed using the DNA according to SEQ ID NO:43 with the primer pairs having DNA sequences according to SEQ ID NO:44/SEQ ID NO:46 and primer pairs having DNA sequences according to SEQ ID NO:45/SEQ ID NO:46. The resulting products (SEQ ID NO:47 and SEQ ID NO:48) were isolated from a 1% agarose gel, digested with Sfi I and ligated into pDAB439. An appropriate clone was identified for each construct, grown up and sequenced thus yielding the plasmids pDAB439/TPE containing SEQ ID NO:47 and pDAB439/TPnoE containing SEQ ID NO:48.

Plasmid pDAB439 was a 7040 base pairs double stranded plant transformation vector composed of the following sequences in clockwise order. The plasmid backbone was derived from pUC19 (Yanish-Perron et al., (1985) Gene 33:103-119). Nucleotides 1 to 2252 of pDAB439 corresponded to the reverse complement of nucleotides 435 to 2686 of pUC19. Nucleotides 2253 to 2271 of pDAB439 had the sequence TGCATGTGTT CTCCTTTTT (SEQ ID NO:52). Nucleotides 2272 to 4264 of pDAB439 were the maize ubiquitin promoter and first intron, and were PCR amplified from genomic DNA of maize genotype B73 (Christensen et al., (1992) Plant Mol. Biol. 18:675-689). Nucleotides 4265 to 4308 of pDAB439 had the sequence GGTACGGCCA TATTGGCCGA GCTCGGCCTC TCTGGCCGAT CCCC (SEA ID NO:53). Nucleotides 4309 to 4576 of pDAB439 corresponded to nucleotides 4420 to 4687 of plasmid pBI101 (Clontech, Palo Alto, CA) followed by the linker GG as nucleotides 4577 and 4578 of pDAB439. Nucleotides 4579 to 4743 of pDAB439 were the reverse complement of nucleotides 238-402 of pUC19. Nucleotides 4744 to 4807 of pDAB439 corresponded to: GCGGCCGCTT TAACGCCCGG GCATTAAAT

GGCGCGCCGC GATCGCTTGC AGATCTGCAT GGG (SEQ ID NO:54).

Nucleotides 4808-5416 of pDAB439 comprised the double enhanced 35S promoter, with nucleotides 5070 to 5416 corresponding to nucleotides 7093 to 7439 of the

5 Cauliflower Mosaic Virus genome (Franck et al., (1980) Cell 21:285-294). Nucleotides 4808 to 5061 of pDAB439 were a duplication of nucleotides 5068 to 5321.

Nucleotides 5062 to 5067 of pDAB439 comprised the linker CATCGA. Nucleotides 5417-5436 of pDAB439 comprised the

10 linker GGGGACTCTA GAGGATCCAG (SEQ ID NO:55). Nucleotides 5437 to 5547 of pDAB439 corresponded to nucleotides 167 to 277 of the Maize Streak Virus genome (Mullineaux et al., (1984) EMBO J. 3:3063-3068). Nucleotides 5548 to 5764 of pDAB439 corresponded to the modified first intron 15 of the maize alcohol dehydrogenase gene (Adh1-S) (Dennis et al., (1984) Nucleic Acids Res. 12:3983-4000). The modification resulted in removal of 343 nucleotides

(bases 1313 to 1656) with bases 1222 to 1312 (intron 5' end) and nucleotides 1657 to 1775 (intron 3' end) of the 20 maize Adh1-S gene remaining. Nucleotides 5765 to 5802 of pDAB439 corresponded to Maize Streak Virus (MSV)

nucleotides 278 to 312, followed by the linker sequence CAG. Both sections of the Maize Streak Virus, hereinafter MSV, sequence comprised the untranslated

25 leader of the MSV coat protein V2 gene, and were interrupted in plasmid pDAB439 by the modified Adh1 intron. Nucleotides 5803 to 6359 of plasmid pDAB439 corresponded to nucleotides 29 to 585 of the phosphinotricin acetyl transferase (BAR) gene of

30 *Streptomyces hygroscopicus* (White et al., (1989) Nucleic Acids Res. 18:1062). To facilitate cloning, nucleotides 34 and 575 of the published sequence were changed from A and G to G and A, respectively. This sequence served as the selectable marker in plant cells. Nucleotides 6360 35 to 6364 comprised the linker GATCT. Nucleotides 6365 to 6635 of pDAB439 corresponded to nucleotides 4420 to 4683

of plasmid pBI101 (Clontech, Palo Alto, CA) followed by the linker sequence AGATCGC. Nucleotides 6636 to 6639 of pDAB439 comprised the linker TCGG. The remaining sequence of pDAB439 (nucleotides 6640 to 7040) corresponded to nucleotides 284 to 684 of pUC19.

EXAMPLE 9

GENERATION OF TRANSGENIC MAIZE PLANTS CONTAINING AND EXPRESSING THE GENES OF INTEREST

10

Type II callus cultures were initiated from immature zygotic embryos of the genotype "Hi-II." (Armstrong et al, (1991) Maize Cooperation Newsletter, pp.92-93). Embryos were isolated from greenhouse-grown ears from crosses between Hi-II parent A and Hi-II parent B or F2 embryos derived from a self- or sib-pollination of a Hi-II plant. Immature embryos (1.5 to 3.5 mm) were cultured on initiation medium consisting of N6 salts and vitamins (Chu et al, (1978) *The N6 medium and its application to anther culture of cereal crops*. Proc. Symp. Plant Tissue Culture, Peking Press, 43-56) 1.0 mg/L 2,4-D, 25 mM L-proline, 100 mg/L casein hydrolysate, 10 mg/L AgNO₃, 2.5 g/L GELRITE, and 20 g/L sucrose, with a pH of 5.8. Selection for Type II callus took place for ca. 2-12 weeks. After four to six weeks callus was subcultured onto maintenance medium (initiation medium in which AgNO₃ was omitted and L-proline was reduced to 6 mM).

The plasmids pDAB439/TPE and pDAB439/TPnoE were transformed into embryogenic callus via helium bombardment. For blasting 140 µg of plasmid DNA was precipitated onto 60 mg of alcohol-rinsed, spherical gold particles (1.5 - 3.0 µm diameter) by adding 74 µL of 2.5 M CaCl₂ H₂O and 30 µL of 0.1 M spermidine (free base) to 300 µL of plasmid DNA and H₂O. The solution was immediately vortexed and the DNA-coated gold particles were allowed to settle. The resulting clear supernatant

was removed and the gold particles were resuspended in 1 mL of absolute ethanol. This suspension was diluted with absolute ethanol to obtain 15 mg DNA-coated gold/mL. Approximately 600 mg of embryogenic callus tissue was

5 spread over the surface of Type II callus maintenance medium as described herein lacking casein hydrolysate and L-proline, but supplemented with 0.2 M sorbitol and 0.2 M mannitol as an osmoticum. Following a 4 h pre-treatment, tissue was transferred to culture dishes containing

10 blasting medium (osmotic media solidified with 20 g/L tissue culture agar (JRH Biosciences, Lenexa, KS) instead of 7 g/L GELRITE (Schweizerhall, South Plainfield, NJ). Helium blasting accelerated suspended DNA-coated gold particles towards and into the prepared tissue targets.

15 The device used was an earlier prototype of that described in US Patent #5,141,131 which is incorporated herein by reference. Tissues were covered with a stainless steel screen (104 μ m openings) and placed under a partial vacuum of 25 inches of Hg in the device

20 chamber. The DNA-coated gold particles were further diluted 1:1 with absolute ethanol prior to blasting and were accelerated at the callus targets four times using a helium pressure of 1500 psi, with each blast delivering 20 μ L of the DNA/gold suspension. Immediately post-

25 blasting, tissue was transferred to osmotic media for a 16-24 h recovery period. Afterwards, the tissue was divided into small pieces and transferred to selection medium (maintenance medium lacking casein hydrolysate and L-proline but having 30 mg/L BASTA (Agrevo)). Every four

30 weeks for 3 months, tissue pieces were non-selectively transferred to fresh selection medium. After 7 weeks and up to 22 weeks, callus sectors found proliferating against a background of growth-inhibited tissue were removed and isolated. The resulting BASTA-resistant

35 tissue was subcultured biweekly onto fresh selection medium. Following gas chromatography/fatty acid methyl

ester, hereinafter GC/FAME, analyses, as described herein, positive transgenic lines were identified and transferred to regeneration media.

Regeneration was initiated by transferring callus tissue
5 to cytokinin-based induction medium, which consisted of Murashige and Skoog salts, hereinafter MS salts, and vitamins (Murashige and Skoog, (1962) *Physiol. Plant.* 15: 473-497) 30 g/L sucrose, 100 mg/L *myo*-inositol, 30 g/L mannitol, 5 mg/L 6-benzylaminopurine, hereinafter
10 BAP, 0.025 mg/L 2,4-D, 30 mg/L BASTA, and 2.5 g/L GELRITE (Schweizerhall) at pH 5.7. The cultures were placed in low light (125 ft-candles) for one week followed by one week in high light (325 ft-candles). Following a two week induction period, tissue was non-selectively
15 transferred to hormone-free regeneration medium, which was identical to the induction medium except that it lacked 2,4-D and BAP, and was kept in high light. Small (1.5-3 cm) plantlets were removed and placed in 150x25 mm culture tubes containing SH medium (SH salts and vitamins
20 (Schenk and Hildebrandt, (1972) *Can. J. Bot.* 50:199-204), 10 g/L sucrose, 100 mg/L *myo*-inositol, 5 mL/L FeEDTA, and 2.5 g/L GELRITE (Schweizerhall), pH 5.8). Plantlets were then transferred to 10 cm pots containing approximately 0.1 kg of METRO-MIX 360 (The Scotts Co. Marysville, OH)
25 in the greenhouse as soon as they exhibited growth and developed a sufficient root system. They were grown with a 16 h photoperiod supplemented by a combination of high pressure sodium and metal halide lamps, and were watered as needed with a combination of three independent Peters
30 Excel fertilizer formulations (Grace-Sierra Horticultural Products Company, Milpitas, CA). At the 3-5 leaf stage, plants were transferred to five gallon pots containing approximately 4 kg METRO-MIX 360. Primary regenerants were self- or sib-pollinated, or outcrossed to either
35 elite inbreds or transgenic plants after an additional 6

- 10 weeks in the 5 gallon pots. R₁ seed was collected at 40-45 days post-pollination.

Embryogenic callus material containing the genes of interest was maintained as described herein. Continuous
 5 production of somatic embryos, which make up a large portion of embryogenic callus, was performed by transferring the callus tissue every two weeks. While the somatic embryos continued to proliferate, they usually remained in an early stage of embryo development
 10 because of the continued presence of 2,4-D in the culture medium. Somatic embryos could be regenerated into plantlets when callus was subjected to the regeneration procedure described herein. During regeneration, somatic embryos formed roots and a shoot, subsequently ceasing
 15 development as an embryo.

Somatic embryos were made to develop as seed embryos by growing embryogenic callus on MS medium containing 6% (w/v) sucrose. The callus was grown for 7 days and then somatic embryos were individually transferred to MS
 20 medium with 6% sucrose and 10 μ M abscisic acid, hereinafter ABA.

EXAMPLE 10

SOUTHERN ANALYSIS OF TRANSFORMED CALLUS AND PLANT TISSUES

25

BASTA resistant lines transformed with various plasmids were characterized by Southern analysis to confirm the presence of the transgene using a DNA probe specific for the coding region of the gene of interest.
 30 DNA from leaf material was analyzed.

Leaf material from plants was harvested at the 6-8 leaf stage. Genomic DNA was prepared from lyophilized tissue as described by Saghai-Maroo et. al. ((1984) *Proceed. Nat. Acad. Sci. USA* 81:8014-8018). Eight μ g of
 35 each DNA was digested with the restriction enzyme(s) specific for each plasmid construct using conditions

suggested by the manufacturer (Bethesda Research Laboratory) and separated by electrophoresis on a 0.8% agarose gel. The DNA was then blotted onto nylon membranes as described by Southern ((1975) J. Mol. Biol., 98:503-517). The radioactive probe was then hybridized to the genomic DNA on the blots in 45 mL of minimal hybridization buffer [10% polyethylene glycol, 7% SDS, 0.6x SSC, 10 mM sodium phosphate, 5 mM EDTA and 100 µg/mL denatured salmon sperm DNA] overnight at 60° C. After hybridization, blots were washed at 60° C in 0.25X SSC and 0.2% SDS for 45 min., blotted dry and exposed to XAR-5 film (Kodak) overnight on two intensifying screens (DuPont).

All lines analyzed showed at least one copy of the expected sized fragment indicating a complete copy of SCAB-TP1 gene.

EXAMPLE 11

SCAB-TP1 EXPRESSION IN MAIZE

For RNA isolation, the tissue was pulverized using a Bessman Tissue Pulverizer (Spectrum Medical Industries, Houston, TX). RNA was extracted from the frozen powder using RNEASY Plant mini kits (Qiagen) according to the manufacturer's instructions. For expression analysis (Northern analysis), 5 µg RNA per sample was fractionated by electrophoresis in non-denaturing 10 mM NaPO₄ pH 6.8, 1.0% agarose gels. The volume of sample containing said RNA was dried using a SAVANT SpeedVac (Savant Instruments, Inc., Farmingdale, NY), resuspended in 8 µL water, and denatured with an equal volume of 2x sample buffer (40 mM NaHPO₄, pH 6.8; 10 mM EDTA, 6% formaldehyde, 50% formamide) and heated to 68° C for 15 minutes. The denatured sample was chilled on ice and 4 µL loading buffer (50% glycerol, 10 mM EDTA, 5 mM NaPO₄, 0.25% bromophenol blue) was added. The samples were loaded on

the gel and electrophoresed for 3 h at 60 V in 10 mM phosphate buffer. RNA was transferred from the gel to GENESCREEN PLUS membrane (NEN Research Products, Boston, MA) by capillary transfer with sterile water as the transfer medium. Following transfer, the RNA was crosslinked to said membrane by UV (STRATALINKER, Stratagene Cloning Systems, Inc., La Jolla, CA).

The RNA blot was prehybridized for 3 h at 42° C in hybridization buffer (50 mM sodium phosphate, pH 6.5, 0.8 M sodium chloride, 1 M EDTA, 0.2% sodium dodecyl sulfate, 0.05% bovine serum albumin, 0.05% Ficoll Type 40, 10% dextran sulfate). A hybridization probed specific for the antibody made against the transit peptide was labelled with 50 µCi of α -³²P-dCTP (NEN Research Products) using READY-TO-GO labelling beads (Pharmacia, Piscataway, NJ) according to the manufacturers instructions and purified over NUCTRAP columns (Stratagene). The labeled probe was denatured by boiling for 5 min, chilled on ice for 5 min, and added directly to the prehybridization blots. Hybridization was done in SEAL-A-MEAL bags (Dazey Corp., Industrial Airport, KA), at 42° C for 16 h. Blots were washed 6 times for 0.5 h each in large excess of prewarmed washing solution (20 mM NaPO₄, pH 6.5, 50 mM NaCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate) at 60° C. The blot was exposed to a phosphor storage screen, scanned on a Molecular Dynamics Personal PHOSPHORIMAGER (Molecular Dynamics, Inc., Sunnyvale, CA) for analysis.

For analysis of R₀ plants, leaf tissue (~ 0.2 g) was harvested from plants transformed with pDAB439/TPE and pDAB439/TPnoE. RNA was extracted, fractionated, blotted to membranes and probed as described above. Lines that were positive for transgenic RNA at the callus stage were found to be positive at the R₀ plants stage. These data indicated that the transgene was integrated and expressing mRNA related to the gene of interest.

To examine protein expression, SCAb-TP1 protein was purified from transformed maize callus tissue using the Qiagen Ni-NTA kit. Callus tissue (0.75-1.45g) was ground with 400-600 μ L 1X binding buffer containing 1mM Phenylmethylsulfonyl fluoride (PMSF) using a plastic tissue grinder and a small amount of sand. The lysate was then spun in a microfuge for 10 min to pellet cellular debris. The supernatant was filtered through a 0.2 μ m syringe filter and applied to Ni-NTA spin column (Qiagen). The column was washed with 600 μ L wash buffer and eluted with 400 μ L elution buffer. The eluted material was then precipitated with acetone or concentrated with 3000 mw micro spin column (Amicon, Beverly, MA) and buffer exchanged with 10 mM phosphate. The concentrated protein was then run in 4-20% SDS-PAGE gels (Integrated Separation Systems, Natick, MA) and Western analysis were performed as described herein. A MRGS 6X HIS MAb preparation (Qiagen) was used as primary antibody. Detectable levels of SCAb-TP1 protein were observed in lines transformed with pDAB439/TPE and pDAB439/TPnoE.

EXAMPLE 12

EFFECT OF SCAB-TP1 EXPRESSION ON ENDOGENOUS Δ -9 DESATURASE LEVELS IN TRANSFORMED MAIZE

The effects of SCAb-TP1 protein expression on endogenous levels of Δ -9 desaturase were examined using transformed maize callus tissues via Western analyses as described herein. Polyclonal antibodies immunologically reactive against mature Δ -9 desaturase were used as the primary antibody. Western data indicated that expression of SCAb-TP1 in callus tissue caused about 70% reduction in the levels of Δ -9 desaturase compared with control callus lines that were transformed with unrelated genes. Protein analysis on leaf tissues of R_0 plants also showed

reduced levels (10 to 50%) of Δ -9 desaturase when compared to control lines. These data indicated a direct correlation between the presence and expression of the transgene and reduced Δ -9 desaturase levels (Table 1).

5

Table 1. Comparison of Δ -9 desaturase levels in maize lines transformed the pDAB439/TPE and pDAB439/TPnoE relative to control.

Line	SCAb-TP1 Protein	Reduced Δ -9 desaturase
TPE-04	+	yes
TPE-05	+	yes
TPE-06	+	yes
TPE-07	+	yes
TPE-08	nd	yes
TPE-09	+	yes
TPE-10	+	yes
TPnoE-01	nd	yes
TPnoE-04	nd	yes
TPnoE-05	+	yes
TPnoE-07	+	yes
TPnoE-11	+	yes
TPnoE-12	nd	yes

10 nd: not determined

Fertile plants containing pDAB463/TPnoE were crossed with inbred CQ715 HO-I (Mycogen Seeds, San Diego, CA) as pollen donors and seed were obtained. These seed were planted and molecular analyses were performed on leaf samples to determine the presence of SCAB-TP1 gene, SCAB-TP1 protein and their effect on Δ -9 desaturase levels. Reduction in Δ 9-desaturase levels (40-70%) was found to correlated with the presence of the transgene and SCAB-TP1 expressed therefrom.

20

EXAMPLE 13

Antibody-Mediated Down-Regulation of Maize Palmitoyl-ACP Thioesterase (PTE)

25

Antibodies directed against the chloroplast transit peptide domain of the PTE protein are used in this

example to block import of PTE into the chloroplast where early steps of fatty acid biosynthesis occur.

A. Analysis of PTE Transit Peptides from Various Maize Genotypes.

Successful application of antibody mediated downregulation requires that the amino acid sequence of the transit peptide be highly conserved between the maize lines used for transformation and regeneration in the laboratory, and the elite maize lines into which the genes will be introgressed for commercial development.

DNA sequences encoding the transit peptide domain of PTE were amplified from eight different corn varieties: CS608, CQ806, HO1, HiII, OQ414, HO601, 2XH753 and 2XH755.

The deduced protein sequences were almost identical among all lines, differing only by the absence of one or two amino acids of the 90 amino acids which comprise the transit peptide. These theoretical amino acid sequences were used to design a region for antibody targeting.

B. Design of Antibodies Targeted to the maize PTE Transit Peptide

The amino acid sequence of PTE precursor protein from maize line CS608 was analyzed to identify a region on the transit peptide portion that exhibits high antigenicity and surface probability. A sequence (PGSPAPAAPKNGGLGERPESLDVRG) (SEQ ID NO:56) extending from amino acid residue number 12 to 35 was selected. The sequence information was sent to Zymed Laboratories Inc. for peptide synthesis and polyclonal antibody production in two rabbits.

C. Baculovirus expression of PTE precursor protein

The gene coding for PTE precursor (including signal peptide) was cloned into the baculovirus transfer vector pAcSG2 (PharMingen, San Diego, CA). A cotransfection was

performed in a 6-well plate with 6×10^5 Sf9 cells/well. BacPAK6 linear DNA (Clontech, Palo Alto, CA) was used as parental DNA in the cotransfection. Bacfectin reagent was used to facilitate cotransfection (included with
5 BacPAK6 DNA). Transfection media was harvested at 3 and 6 days post-transfection. Sf9 cells (6×10^5 cells/well) were infected with 100 μ l transfection media. After 3 days, cells were harvested and western blots were performed. Blots were probed with rabbit polyclonal
10 antibody generated against the mature PTE protein or to the transit peptide of the precursor protein (rabbit 1 or rabbit 2). The blot probed with antibody to the mature protein showed 2 distinct bands for PTE with or without the transit peptide. Blots probed with antibody to the
15 transit peptide only detected the higher molecular weight form of the PTE containing the transit peptide.

Production of hybridoma cell lines that produce antibodies that bind to the PTE precursor protein,
20 cloning of the antibody genes and single chain antibody gene, and transformation into corn can be carried out as described in foregoing Examples 5 through 9.